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Oxidative Stress and Cell Death in Osmotically Swollen Glial Cells

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OXIDATIVE STRESS AND CELL DEATH IN OSMOTICALLY SWOLLEN GLIAL CELLS

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Crystal E. Stuckey ENTITLED Oxidative Stress and Cell Death in Osmotically Swollen Glial Cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Regulation of normal volume is an important aspect of cell homeostasis. Possible mechanisms which signal volume regulation are increasing the rate of reactive oxygen species (ROS) production and release of ATP for interaction with purinergic receptors. We examined whether an increase in ROS production during cell swelling also led to cell injury of C6 glioma cells. Cells were loaded with 5,6-carboxy-2,7-dihydrofluorscein diacetate (DCFDA) to fluoroscopically measure the rate of cellular ROS production and were perfused with phosphate-buffered saline solutions (PBS) containing 100 μM carbenoxolone to inhibit dye efflux. Cell death was determined cytometrically and by measuring the release of lactate dehydrogenase (LDH) into the culture medium 24 hr after exposing cells for 60 min to isoosmotic or hypoosmotic PBS. Immediately after changing the perfusion solution from isoosmotic to hypoosmotic PBS, the production of ROS increased by 60.2% $\pm$ 19.6, but returned to baseline after 5 min. Increased efflux of ATP was not observed in hypoosmotic conditions. ROS production was not directly activated by endogenously applied extracellular ATP, but ATP increased ROS production in swollen cells. Cells swollen by hypoosmotic solutions had a slight increase in the probability of necrotic cell death. Our data suggest that increased ROS produced by cells swollen during hypoosmotic stress does not lead to significant cell injury in cultured C6 cells.

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I. Introduction and Purpose

Introduction

The redox state of a cell is highly regulated and is determined in part by the ratio of free radicals to antioxidants present within the cell. A predominate class of free radicals is reactive oxygen species (ROS). ROS are highly reactive molecules and are used in many ways by a cell including the regulation of cellular processes [59, 72]. If the ROS levels get out of physiological ranges they can cause cell injury and death. ROS can be produced by several different intracellular sources such as NADPH oxidase complex, mitochondrial electron transport chain, xanthine oxidase, and nitric oxide synthase [2, 21, 68, 104]. Many cell types have been known to increase their production of ROS when they are under mechanical or osmotic stress. Regulation of volume is especially important when the cells are exposed to anisoosmotic conditions. Two potential signals for volume regulation when a cell is exposed to anisoosmotic conditions are ROS and ATP. Volume regulation can be accomplished with activation of ROS, ATP, or a combination of both of these compounds. Activation of volume regulatory processes allows the cell to efflux ions and organic osmolytes which draws water out of the cell. This study was designed to determine if there was an increase in ROS production during hypoosmotic swelling which led to an increase in cytotoxicity, and to determine the role of ATP in the regulation of ROS production.

Cell Volume Regulation

Cell volume regulation is an important aspect of the life of a cell. Cells must be able to regulate their volume in isoosmotic conditions as well as anisoosmotic conditions. The mammalian cell is only protected by a selectively permeable phospholipid membrane that allows water to move freely across the membrane. When the osmotic environment changes in or around the cell, water moves causing the cell to swell or shrink. If too much water moves into the cell it can cause rupturing of the membrane. On the other hand, if too much water leaves the cell, the internal environment will become too concentrated with ions and the cell will not be able to carry out its normal functions. The ways in which a cell is able to control its volume are by changing the intracellular osmolyte content through movement of ions and organic osmolytes across the plasma membrane, anabolism, and catabolism. Among other functions, plasma membrane channels allow the movement of small or charged molecules to move through the plasma membrane. Thus cells use the phospholipid bilayer and proteins embedded within the bilayer to control the environment of the interior of the cell and maintain a dynamic, but steady volume [153].

In steady state conditions, there is a constant risk to cell volume equilibrium due to the asymmetrical distribution of ions and osmotic macromolecules across the cell membrane. The Gibbs-Donnan equilibrium principle states that the sum of the monovalent cations times sum of the monovalent anions on one side of the membrane is equal to the products of the activities of the sum of these ions on the other side of the membrane. Thus, for a simple solution containing only intracellular (i) and extracellular (o) potassium and chloride:

$$[K^+]_i \times [Cl^-]_i = [K^+]_o \times Cl^-]_o \quad [1]$$

Mammalian cells use a system similar to the Gibbs-Donnan system known as the double Donnan principle to control water movement across the membrane. For this system, an extracellular solute, sodium (Na^+), can balance the osmotic pressure of the intracellular macromolecules. In order for this to work, the cell has to be effectively impermeable to this solute. If the solute has some permeability through the cell membrane, the cell must transport this solute out of the cell as fast as it enters the cell. This is accomplished with the Na^+/K^+ pump. Sodium is pumped out of the cell, against its gradient, and potassium (K^+) into the cell. Outward transport of sodium maintains a low intracellular concentration and high extracellular concentration of Na^+. This process moves Na^+ against its electrochemical gradient and is therefore an energy dependent process [82, 102, 119].

If the double Donnan equilibrium is disrupted by anisoosmotic conditions, water will move in or out of the cell causing the cell to swell or shrink, respectively, in order to regain osmotic balance across the plasma membrane. Anisoosmotic changes alter the cell membrane causing membrane blebbing and rapid changes of the actin cytoskeleton [119]. Once most mammalian cells are exposed to a change in osmolarity they activate processes to return the cell to normal size. These processes are known as regulatory volume increase (RVI) and regulatory volume decrease (RVD). Both processes may be activated by a cell's ability to monitor $[Cl^-]_i$ (intracellular chloride) and macromolecule concentrations within the cell [119]. Other processes are important in recognizing changes and maintaining cell volume such as stretch activated channels and changes in the cytoskeleton. The altered ionic concentrations caused by cell shrinking or swelling

can cause a change in catalytic rates, ligand binding abilities, structural conformation, and protein assembly [151, 153]. These are some of the stimuli that cells use to monitor changes in cell volume, but the mechanism of activation of RVI and RVD are not entirely clear. If a cell is unable to regulate its volume by RVI or RVD the cell will die by necrosis or apoptosis [119, 162].

Regulatory Volume Increase

When a cell is introduced to a hyperosmotic environment, the cell will shrink as water moves out of the cell to maintain osmotic balance across the plasma membrane. Once the cell shrinks in hyperosmotic conditions a process known as regulatory volume increase (RVI) may be initiated which increases the size of the cell toward its initial cell volume despite continuous osmotic stress. RVI occurs in two steps; the initial step is fast occurring within minutes and is accomplished with inorganic osmolytes and electrolytes [29]. Within minutes of a hyperosmotic change, there is an influx of Na^+, K^+, Cl^-, H^+, and HCO_3^- ions. Then water follows, increasing the cell size to a normal volume [29, 83, 121, 153, 165]. The cell cannot function properly at this altered ionic concentration; therefore, a second process is activated using organic osmolytes [29]. The movement of organic osmolytes is a slower process that takes hours to days because organic osmolytes are either proteins that have to be synthesized within the cell or small molecules that are brought into the cell via energy-dependent transport [153, 165]. The three primary categories of organic osmolytes used for hyperosomtic volume regulation are polyols such as sorbitol and myoinositol, amino acids such as taurine, and methylamines such as betaine and glyerophosphocholine [29, 153, 165]. These organic osmolytes accumulate

within the cell so that the cell is able to remain at homeostatic size and recover normal ionic concentrations.

Two known mechanisms that regulate volume after cell shrinkage are the Na^+/H^+ exchanger combined with Cl^-/HCO_3^- exchanger and Na^+-K^+-$2Cl^-$ co-transporter [121, 153, 165]. Ehrlich ascites cells and vascular endonthelial cells use the Na^+/H^+ exchanger also acting with the Cl^-/HCO_3^- exchanger [77, 88]. The Na^+/H^+ exchanger is present in C6 cells, but is primarily responsible for maintaining pH and can be activated by an acidic intracellular pH [76, 115, 149]. The Na^+/H^+ exchanger also is activated during RVI. During RVI the kinetics of the exchanger are slightly modified, but according to Mountian et al., ion exchange due to the Na^+/H^+ exchanger is not large enough to effect RVI [76, 115]. The main effector of RVI in C6 cells is the Na^+-K^+-$2Cl^-$ co-transporter which, when activated, causes an increase in the levels of NaCl and KCl within the cell [115, 165]. In C6 cells, increase in intracellular Na^+ enhances activity of Na^+-K^+-ATPase which exchanges Na^+ for K^+. This causes Na^+ to move out of the cell in an overall cyclic manner when working along with the co-transporter [40, 47, 115]. Movement of ions leads to an increase in intracellular KCl and movement of water into the cell which enables the cell to regain original cell volume [40, 83, 115]. Inorganic osmolytes and free organic osmolytes allow the cell to regulate volume quickly, but long term RVI is accomplished using organic osmolytes that are made by the cell during the osmotic stress.

Organic osmolytes also play an important role in RVI. They allow the cell to control both cell volume and intracellular ionic concentrations when exposed to prolonged hyperosmotic conditions. The advantage of organic osmolytes is that they are

nonperturbing solutes that can accumulate to high concentrations within the cell without disrupting cell function [151]. Cells can also undergo large shifts of organic osmolytes without effects on cell structure or function [153]. Myo-insitol is one of many organic osmolytes, such as sorbitol, taurine, betaine, and glyerophosphocholine, that cells use to maintain their volume during hyperosmotic stress [29, 40, 124, 165]. Minami et al. found that changes in organic osmolytes did not alter cellular structure or function. They did this by injecting rats intraperitoneally with hypertonic solutions, removing the rat brains and measuring the accumulation of the Na^+/myo-insitol co-transporter [112]. Expression of the Na^+/myo-insitol co-transporter peaked shortly after exposure and then expression of the co-transporter leveled off to a normal level. The importance of the Na^+/myo-insitol co-transporter is evident in that most glial cells and neurons express the co-transporter [112].

Regulatory Volume Decrease

When mammalian cells are exposed to hypoosmotic solutions they initially swell and then regulate volume. The mechanism which triggers volume regulation is still unclear. Activation of volume regulation could result directly from changes in intracellular ionic strengths, tension on the cell membrane due to cell volume changes, or a combination of both [156]. Volume regulation in the cell is generally dependent on efflux of K^+, Cl^-, and organic osmolytes. Charged or small particles exit the cell via protein channels and water follows the electrolytes resulting in decreased volume of the cell, a process known as regulatory volume decrease (RVD). Activation of the volume regulatory channels is a rapid process because the channels are permanently located on

the membrane or stored in submembrane cytoplasmic vesicles [153]. For RVD to be effective, ionic fluxes must be electroneutral [23]. As the cell releases K^+ into the extracellular space, it also fluxes an anion, Cl^-, at the same rate either by two separate transporters or a single co-transporter. One pathway is the electroneutral KCl co-transporter (KCC) which is used by cells that have a slow onset of RVD, a high resting Cl^- conductance and low membrane potential [127]. The second pathway for KCl efflux is the conductive pathway. This is used by cells such as astrocytes which demonstrate a much faster RVD and have a low basal Cl^- permeability [48, 127, 133]. During RVD, astrocytes increase their permeability to Cl^- so much that it surpasses their permeability to K^+. Potassium is then the rate limiting step in RVD. This was revealed by Pasantes-Morales et al. by pharmacologically increasing cation permeability and showing greatly accelerated RVD [127].

Chloride can exit the cell through a channel known as the volume-sensitive outwardly rectifying (VSOR) Cl^- channel. Characteristics of this channel include activation by intracellular ATP and sensitivity to Mg^{2+} that is free in the cytosol [120]. A critical activator of VSOR Cl^- channels during cell swelling, is H_2O_2 produced via NADPH oxidase [156, 163]. Regardless of the means of activation, the chloride current activated by cell swelling is relatively similar in all cell types. The Cl^- current established by VSOR Cl^- channels is outwardly rectifying and demonstrates depolarization-dependent inactivation [156].

RVD is also accomplished using organic osmolytes such as free amino acids, polyhydric alcohols, and methylamines. The common amino acids effluxed from cells in the brain during RVD are taurine, glutamate, and aspartate [141]. Taurine efflux from

astrocytes during hypoosmotic exposure is mediated by a Cl^- dependent diffusion pathway and not a reversal of the energy dependent taurine carrier which brings taurine into the cell [141]. The diffusion pathway for amino acids is permeable to acidic amino acids and those that are neutral (α- and β-amino acids), it is not permeable to basic amino acids such as arginine and lysine [126, 128, 141].

Similar to astrocytes, C6 cells use organic osmolytes to regulate their volume, though they cannot account for RVD alone. The amino acids that are effluxed during hypoosmotic exposure are taurine, aspartate, glutamate, and alanine. According to Mountain et al., there is a low intracellular concentration of amino acids in C6 cells and consequently a small release of these compounds [116].

Necrotic Volume Increase

When a swelling cell is unable to regulate its volume it eventually bursts, a process termed necrotic volume increase (NVI). Swelling during NVI occurs in cytoplasmic organelles as well as the cytoplasm itself [25]. In a whole animal, rupturing of cells causes an inflammatory response in the surrounding tissue [17, 122]. The irreversible step of NVI is ATP depletion. In ROC-1, a rat oligodendrocyte-rat glioma hybrid, irreversible depletion of ATP below 50% causes necrotic cell death [80]. ATP depletion also causes the other characteristic signs of necrosis such as bleb development, cell swelling, plasma membrane disruption and increased intracellular Na^+ concentrations [17, 18, 80, 122].

Apoptotic Volume Decrease

Apoptosis is programmed cell death that occurs in most cell types [161]. Apoptosis occurs in multi-cellular organisms triggered by a set of morphological, biochemical or genetic characteristics [25, 26, 103, 161]. This process allows a cell to be eliminated from a tissue without disrupting the surrounding tissue via inflammation [25, 93]. Apoptosis is an ATP-dependent pathway that allows an organism to get rid of old or unwanted cells. Apoptosis requires high ATP levels, and low levels of intracellular Na^+ [17, 97, 123]. Once apoptosis is activated, the cells undergo characteristic changes which are cell shrinkage (apoptotic volume decrease), nuclear condensation, cleavage of DNA, RNA, and proteins by endonucleases, mitochondrial depolarization, and eventual blebbing of the cell membrane causing apoptotic body formation [25, 26, 93].

One of the first events that occur when a cell undergoes apoptosis is apoptotic volume decrease (AVD). The channels activated during AVD are similar to the channels activated to facilitate RVD during exposure to hypoosmotic media [161]. If volume reduction is prevented by blocking VSOR Cl^- channels or volume regulatory K^+ channels, apoptosis is prevented [93, 103, 161]. The difference between RVD and AVD is that RVD occurs when cells are exposed to hypoosmotic solutions and is used to regulate its volume and AVD occurs in isoosmotic solutions when a cell is destined to die [143]. If a cell is to undergo apoptosis then it must override or turn off the cell's RVI mechanism as it continues to shrink.

Cell death via apoptosis in C6 cells, as well as other glial cell lines, seems to be mediated by phosphorylation of ERK and an increase in p53 [35]. Hydrogen peroxide causes the phosphorylation of ERK through the growth factor receptor and Ras signaling

pathways [96]. Cells exposed to H_2O_2 have increased p53 expression that correlates with apoptotic cell death. When glial cells are exposed to ROS, cytochrome-c is released from mitochondria, possibly due to mitochondrial membrane depolarization. This release activates MDM-2 which is a caspase-3-like molecule that regulates p53. MDM-2 is cleaved causing it to lose its ability for degradation of p53, thus there is an increase in p53. p53 which is normally located in the nucleus, moves to the cytoplasm, mainly in the mitochondria where it activates caspases that leads to apoptosis within the cell [24, 87].

Redox Reactions

Oxygen is a critical part of life for higher organisms. It is necessary for aerobic respiration which provides the greatest amount of cellular energy for most cells. While producing energy aerobic respiration also can produce free radicals as a byproduct. Free radicals are molecules that have an unpaired electron and typically a carbon, oxygen, or nitrogen base. Within the mitochondria, oxygen free radicals are produced at complex I and III of the electron transport chain [21]. Other sites of free radical production within the cell are NADPH oxidase complex, xanthine oxidase, and nitrous oxide synthase. Oxygen radicals react readily with metals, oxidants, and reductants yielding a gamut of reactive species. Not all of the products formed by these free radical reactions are in themselves free radicals [92]. For example, hydrogen peroxide (H_2O_2), which is not a free radical, is formed when the free radical, superoxide, interacts with the enzyme superoxide dismutase (SOD) [55, 137]. Reactive oxygen species (ROS) are molecules that are made from oxygen free radicals and are highly reactive.

Oxygen

Redox reactions are a group of reactions in inorganic chemistry that cause the oxidation and reduction of reactants. Some redox reactions occur via the movement of electrons. A molecule is oxidized as it gains an electron and it is reduced as it loses an electron in two half-reactions, as one molecule gives an electron to another molecule. The redox potential, or the molecules electronegativity E°, is the affinity that a substance has for electrons compared to the affinity for electrons of hydrogen. The direction in which the reaction proceeds depends on the electronegativity of the reaction; the more positive the E° the more readily an oxidation reaction occurs. Redox potentials for the addition of electrons to oxygen are as follows:

$$O_2 + e^- \rightarrow {}^{\cdot}O_2^- \qquad E° = -0.33\ V \qquad [2]$$

$${}^{\cdot}O_2^- + e^- + 2H^+ \rightarrow H_2O_2 \qquad E° = +0.89\ V \qquad [3]$$

$$H_2O_2 + e^- + H^+ \rightarrow H_2O + HO^{\cdot} \qquad E° = +0.38\ V \qquad [4]$$

$$HO^{\cdot} + e^- + H^+ \rightarrow H_2O \qquad E° = +2.31\ V \qquad [5]$$

In the first equation above, the E° for the oxidation of oxygen is negative so a single electron is not easily given to molecular oxygen to make superoxide. But, adding the second, third and fourth electrons as seen in the last three equations is favorable [58]. Once superoxide is formed it can spontaneously act as an oxidant (+ 0.89 V) by adding an electron or a reductant (+ 0.33 V) by donating an electron depending on the redox potential of the total reaction [21, 58, 169]. Many cells use superoxide to make other forms of ROS within the cell. They are able to first make the superoxide and then use the superoxide to act as oxidants or reductants as the cell needs to make compounds such as H_2O_2.

ROS

Reactive oxygen species (ROS) are a broad group of reactive molecules within the cell. Most of the molecules that make up this group are free radicals. There are a few ROS that originate from oxygen free radicals, but are not in and of themselves free radicals, such as H_2O_2 [92]. Examples of ROS are hydrogen peroxide (H_2O_2), superoxide ($\dot{O}_2^-$), hydroxy radical ($\dot{O}H$), and nitric oxide (NO) [58]. ROS act within the cell as a second messenger, but the biological role of each ROS is dependent on its electronegativity. Relatively speaking H_2O_2 and superoxide are less reactive than hydroxy radical and therefore have different roles within the cell. Superoxide and H_2O_2 act as better cellular messengers because they do not react as readily with the first organic compound that they come in contact with. Hydroxy radical, on the other hand, has a greater potential to cause cellular damage because it readily reacts upon initial contact of an organic compound.

The most common ROS produced within the cell are superoxide molecules. These free radicals do not readily move through lipid membranes because of their negative charge. The production of superoxide is spontaneous during cell metabolism, especially in electron rich environments, such as the electron transport chain [21, 118]. By itself, superoxide is only moderately reactive, but it is readily reduced or oxidized into molecules that are highly reactive. Superoxide is the starting molecule for the formation of many different ROS shown below [21].

$\dot{O}_2^- + \dot{O}_2^- + 2H^+ \rightarrow H_2O_2 + O_2$	Dismutase Reaction	[5]
$\dot{O}_2^- + H_2O_2 \rightarrow \dot{O}H + OH^- + O_2$	Harbor-Weiss Reaction	[6]
$Fe^{2+} + H_2O_2 \rightarrow \dot{O}H + OH^- + Fe^{3+}$	Fenton Reaction	[7]

$\dot{O}_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$ Fenton Reaction [8]

Though superoxide is the most common form of ROS produced in the cell, it is not commonly used as a cellular signal. Superoxide is converted to other forms of ROS by the cell to be used as a cellular signal. The form of ROS that is most often used as an intracellular signal is H_2O_2. Two molecules of superoxide spontaneously convert to H_2O_2 and molecular oxygen (equation 5), but this reaction is accelerated by superoxide dismutase (SOD) [118]. Hydrogen peroxide is not a radical, has no charge, and is similar in size to molecular oxygen. These characteristics allow H_2O_2 to freely move across the membrane [118]. Once H_2O_2 enters the cell it can readily react with cysteine residues, allowing H_2O_2 to act as a second messenger in many cellular processes [92]

Another form of ROS produced in cells is hydroxy radical. This molecule is formed when H_2O_2 oxidizes a transition metal (equation 7) or when H_2O_2 reacts with superoxide (equation 6). Superoxide reacts with H_2O_2 via the Haber-Weiss reaction, the sum of equations 7 and 8 [21, 58, 118]. This reaction uses a transition metal catalyst to convert superoxide and H_2O_2 to hydroxy radicals [118]. Hydroxy radicals are very short lived typically and react with the first molecule they contact. Due to its reactivity, $\dot{O}H$ has the greatest potential of all the ROS to cause cellular damage [118]. Hydroxy radical can irreversibly cause lipid peroxidation, DNA damage, and protein oxidation [58].

Other ROS such as nitric oxide (NO) are formed independently of the equations above. Nitric oxide is produced by nitric oxide synthase (NOS). NADPH donates electrons to the amino-terminal of L-arginine forming L-citrulline and NO. NO reacts similarly to superoxide in that it is not readily reactive with biomolecules, but reacts with other free radicals [118]. When NO reacts with free radicals it generates less reactive

molecules which allows NO to act like a free radical scavenger. At physiological concentrations NO acts as a second messenger within the cell. Similar to H_2O_2, NO can pass through the membrane thereby transferring signals to neighboring cells. But, if NO is produced in high amounts with superoxide the two molecules will combine to form peroxynitrate. Peroxynitrate reacts with CO_2 or may get protonated forming peroxyocarboxylate and peroxonitrous acid, respectively both of which are toxic to the cell by attacking tyrosine residues in proteins [118].

Once a free radical is formed by a cell it continues to react and form new free radicals until it comes to interact with another free radical or an antioxidant. When the free radical comes in contact with another atom, the extra electron on the free radical attracts an electron from the other atom. This forms a stable compound and a free radical that then reacts with another atom. The series of reactions that continues to form new free radicals is known as the propagation phase. If two free radicals react with each other or if a free radical interacts with an antioxidant, then the propagation phase is terminated.

Antioxidants

The redox state within a cell is highly regulated. Free radicals are important for cellular functions, but in high concentrations they can harm the cell. One way in which the cell protects itself from the harmful effects of free radicals is by producing antioxidants. Antioxidants bind with free radicals within the cell and render them ineffective and unreactive. The effectiveness of an antioxidant is dependent on the stability of the free radical it forms.

A common lipid soluble antioxidant in mammalian cells is vitamin E. Vitamin E effectively protects DNA from free radical attack by scavenging lipid peroxyl radicals and terminating the propagating redox chain reaction that produces DNA-damaging products. Another protective action of vitamin E is via direct inactivation of ROS [13]. Vitamin E prevents free radical damage by transferring hydrogen to the propagating chain. This creates a vitamin E free radical, which is stable. Free radicals formed by the antioxidants must be more reactive with other free radicals than they are with biological molecules. This is important so that the free radicals formed by the antioxidants do not cause cellular damage. Once the cellular free radical reacts with an ascorbate radical (asc·) the reaction is terminated [170]. For example, trolox C, a vitamin E analogue, donates a hydrogen atom from the hydroxyl group of the chromanol ring on the trolox C molecule. This hydrogen reacts with the propagating free radicals to yield a phenoxyl radical. The phenoxyl radical oxidizes ascorbate and other biomolecules to radicals [158].

Of the vitamin E analogues, trolox C is the most hydrophilic. This property is important for trolox C to be sufficiently soluble in aqueous solutions to allow it to move into the cell. Trolox C has a structure similar to the most common vitamin E analogue in mammals, α-tocopherol [158].

Antioxidants scavenge for free radicals within the cell by forming stable free radicals. The free radical that the antioxidant forms then reacts with other free radicals found within the cell. However, when the antioxidant is present at high concentrations within the cell the scavenging free radicals formed by the antioxidant can themselves cause damage to the cell. The way in which the antioxidant damages the cells is it acts

as a pro-oxidant, oxidizing DNA, proteins and lipids. High concentrations of antioxidants can damage the cell in similar ways as high concentrations of ROS. Vitamin E, like many other antioxidants, has been shown to act as a pro-oxidant when present in high concentrations [158]. Vitamin C (ascorbate) is a water-soluble antioxidant that scavenges reactive oxygen and nitrogen species [13]. Ascorbate forms an ascorbate radical ($asc^{\cdot -}$) which reacts with other free radicals within the cell terminating the propagation of the free radials and protecting the cells.

Biochemical Importance

Depending on the redox state of the cell, ROS can either act as intracellular messengers or produce harmful effects in cells. Most ROS are good signaling molecules because they are easily synthesized, highly diffusible, easily degraded, and present in most cell types [59]. Thus, ROS act as intracellular signals in areas such as activation of transcription factors, inducing apoptosis, activating or deactivating ion channels, and inducing an immune response [92, 96, 108, 110, 129, 143, 144]. Because of the reactivity of ROS, antioxidant systems within the cell are needed to protect the cell from the potentially harmful effects of ROS. When antioxidant systems within the cell are not able to maintain physiological concentrations of ROS cell toxicity may occur.

ROS are unique as intracellular signals because they are highly reactive, interacting with most organic molecules within the cell [129]. Normally intracellular signals are very specific, reacting with one element of a signaling pathway. But ROS are able to act as intracellular signals by modifying the redox potential of the cell [92]. The intracellular redox potential moves towards oxidation when the level of the ROS

increases or the level of antioxidants decreases below physiological range. This may cause irreversible damage to macromolecules within the cell [58] including lipid peroxidation, and damage to DNA [3, 36, 101, 106]. Some diseases have been associated with altered redox state including Alzheimer's disease, Parkinson's disease, enteric disorders, diabetes insipidus, diabetes mellitus, Huntington's disease, and amyotrophic lateral sclerosis [6, 66, 115, 140].

Sources of ROS

The understanding of free radical production and function within cells has changed over the past few years. Free radical production was first discovered as a byproduct of aerobic respiration, but recent research has established that free radicals are a necessary part of cell function [43]. In order for ROS to act as a cell signal their production must be regulated by the cell, they must have an action on the same or nearby cell, and the ROS must be removed to eliminate the signal [62]. The major sites of intracellular ROS production that will be discussed are NADPH oxidase, mitochondria, xanthine oxidase, and nitric oxide synthase.

NADPH Oxidase Complex

NADPH oxidase was discovered as the location of ROS production in phagocytic cells. This production of ROS is known as a respiratory burst, and is used by the phagocytic cell to generate powerful oxidants as an immunological mechanism. NADPH oxidase reduces molecular oxygen to superoxide which destroys microorganisms [45].

NADPH oxidase is composed of two pools of peptide components located in the cytosol and the other two are membrane components. When the components of the complex are separated, NADPH oxidase is dormant, but when stimulated the components come together at the plasma membrane and the complex is activated. The cytosolic components are made up of $p67^{phox}$ / $p40^{phox}$ and GTPase Rac1/Rac2 [56]. The membrane bound components are $p47^{phox}$ and $gp91^{phox}$. Two of the components, $p47^{phox}$ and $p67^{phox}$, may have NADPH and FAD binding sites, but the exact function of the remainder of the components is still unknown [78]. In phagocytic cells the cytosolic components move to the plasma membrane when cells are exposed to microorganisms or inflammatory mediators. When the cytosolic components bind to the membrane components at the membrane, NADPH oxidase is activated [125]. Arachidonic acid (AA) interacts with the cytosolic component, $p47^{phox}$ and causes it to undergo a conformational change. This change in $p47^{phox}$ opens up a binding site for interaction with $p22^{phox}$, a membrane-bound component. Phosphorylation of $p47^{phox}$ also plays a role in its activation. The two membrane bound components come together to form a stabilized complex known as flavocytochrome b_{558}. Once this complex is assembled it acts as the catalytic core that allows for the transfer of electrons from NADPH to molecular oxygen thus reducing extracellular O_2 to $O_2^{\cdot -}$ [57]. Nonenzymatic $O_2^{\cdot -}$ dismutation quickly occurs forming H_2O_2; however, superoxide dismutase can accelerate this reaction. Most superoxide dismutases are found outside the cell; therefore, the majority of the conversion of $O_2^{\cdot -}$ to H_2O_2 takes place in the extracellular space [56].

Like many other cell types, astrocytes use NADPH oxidase to produce ROS [100]. Under hypoosmotic conditions NADPH oxidase is activated [135]. Swelling of

the astrocytes activates PKC which then causes rapid serine phosphorylation of the $p47^{phox}$ component [135]. Hypoosmotic swelling also increases intracellular calcium levels which also play a role in the activation of NADPH [2]. This occurs because swelling of astrocytes is sufficient to induce serine phosphorylation of $p47^{phox}$ causing NADPH-dependent ROS production [2, 135].

Mitochondria

A second major source of ROS is the electron transport chain (ETC) in the mitochondria. The ETC oxidizes the reduced cofactors, $FADH_2$ and NADH, by transferring electrons to oxygen through a series of steps. The free energy from the oxidation-reduction reactions in the ETC is used to form a membrane pH gradient across the inner mitochondrial membrane which provides energy for ATP synthesis [10, 44]. During this process, some molecular oxygen may be reduced thus forming a superoxide. Approximately one to two percent of oxygen consumed systemically is converted to ROS in the mitochondria [146].

The ETC is made up of 4 complexes, complex I, II, III, and IV. Electrons are carried from the Krebs Cycle by NADH and $FADH_2$. NADH moves 2 electrons to complex I which then moves them to the ubiquinone pool in the intermembrane space. Complex II receives electrons from $FADH_2$ and moves them to the ubiquinone pool by an iron-sulfur center. The electrons continue from the ubiquinone pool to complex III where they are transferred to cytochrome c and then to complex IV. When the electrons reach complex IV they are transferred to molecular oxygen. Electrons are transferred from reactants with more negative reduction potentials to an electron acceptor that has a more

positive reduction potential. Each of these complexes is only capable of transferring one electron at a time to the next site along the ETC.

Similar to NADPH oxidase, mitochondrial ETC can form superoxides by reducing molecular oxygen. The two major sites in the ETC of the mitochondria that are responsible for the monoelectronic reduction of oxygen are complexes I and III. These two complexes have a slight tendency to transfer electrons to oxygen instead of the next electron acceptor in the ETC causing the reduction of molecular oxygen and the formation of superoxide radical. The rate of the ROS production in the mitochondria is highly dependent on the mitochondrial membrane potential. When the respiratory rate decreases because of a decrease in intracellular ADP concentration, there is an increase in the mitochondrial membrane potential which causes an increase in the production of ROS [111].

Only a single electron can be transferred at a time from an electron donator to the electron acceptor. There are two pools of ubiquinol, one accepts electrons from complex I and the other accepts electrons from complex III. Ubiquinol can carry two electrons from complex I and III; transferring the electrons one at a time to the next electron acceptor. Movement of one electron at a time to components such as NADPH, ubiquinone, and cytochrome c allows for free radical formation. Specifically this occurs as NADH dehydrogenase and ubiquinone transfers one electron to an iron-sulfur cluster and forms semiquinone. The semiquinone then oxidizes forming superoxide [8].

Most ROS production during normal respiration occurs at complex I, NADH-ubiquinone oxidoreductase. Complex I is responsible for catalyzing the transfer of electrons from NADH to ubiquinone. During aerobic respiration, NADH gives two

electrons and two protons to complex I. Complex I then transfers the electrons to ubiquinone, a lipid soluble carrier. The energy released in the transfer of electrons is preserved by the movement of four protons across the membrane to the mitochondrial intermembrane space. This movement of protons sets up a proton gradient across the mitochondrial inner membrane. During this process some electrons are transferred to molecular oxygen forming superoxide.

Another location of ROS production in the mitochondrial ETC is complex III, ubiquinol-cytochrome c oxidoreductase. This complex is involved in the movement of two electrons and four protons. Complex III moves one electron at a time from ubiquinol, which contains two electrons that it can donate, to cytochrome c, a water-soluble electron carrier that is in the intermembrane space. During the movement of electrons to cytochrome c, some are transferred to molecular oxygen to make superoxide.

Xanthine Oxidase

Another major source of ROS is xanthine oxidase. Xanthine oxidase contributes very little to ROS production in a normally functioning cell, but has been shown to be a source of ROS during oxidative stress [43]. Xanthine dehydrogenase (XDH) and xanthine oxidase (XO) are reversibly convertible within the cell. XDH can be converted to XO by oxidizing eight cysteine residues to four cysteine residues with pyrimidines or oxidized glutathione [19, 64]. This reaction can be reversed by a reducing agent like dithiothreitol [7]. XDH and XO can be used to reduce NAD or molecular oxygen, respectively. The redox state of the cell oxidizes or reduces the enzyme forming XO or

XDH, respectively. Thus, when cells are at an oxidized redox potential, XO will be formed and cause formation superoxide from molecular oxygen [63].

The reaction of xanthine oxidase begins with the conversion of hypoxanthine which is converted to xanthine and uric acid. When xanthine is reduced to uric acid, two electrons are given to xanthine oxidoreductase (XOR) which reduces molybdopterin (Mo) VI to Mo(IV) forming XOR^{2e^-}-Mo(IV). When xanthine oxidase is fully reduced it has the ability to donate six electrons. The first four electrons are donated to 2 oxygen molecules to make hydrogen peroxide (H_2O_2). The last two electrons are donated one at a time to oxygen creating superoxide ($O_2^{\cdot -}$). Thus fully reduced xanthine oxidase has the capability of making two H_2O_2 and two $O_2^{\cdot -}$ [63, 69].

The production of ROS via xanthine oxidase is largely due to oxidative stress. The exact mechanism for the activation of xanthine oxidase is unclear, but it is proposed by Kayyali et al.'s work that phosphorylation of XOR in hypoxic situations is associated with enhanced enzyme activity [22, 81].

Nitric Oxide Synthase

Nitric oxide synthase (NOS) is a dimeric enzyme that is only active when the two subunits come together. Activated NOS is capable of making the free radical nitric oxide (NO). This enzyme works by oxidizing the terminal guanidinium nitrogens of L-arginine forming NO and L-citrulline. This reaction is a two step process. In the first step L-arginine is oxidized with two electrons forming N-hydroxy-L-arginine (NOHA). The second step takes NOHA and further oxidizes it to form NO and L-citrulline. The redox reaction uses the enzyme NOS to transfer electrons from NADPH to $O_2^{\cdot -}$. There are three

main isoforms of NOS, endothelial (eNOS), neuronal (nNOS) and immunological/inducible (iNOS). Each of these comes from individual genes, are regulated separately and are located in different places within the cell [58, 154]. Studies have shown that eNOS and nNOS are 60% identical, eNOS and iNOS are 50% identical, and iNOS and nNOS are 50% identical [60]. Another type of NOS is mitochondrial NOS (mtNOS) which is similar in form and function to nNOS. Stimulation of mtNOS changes the rate of oxygen free radical production in the mitochondrion [46, 142].

Knowles et al. [89] found that NO synthases in the normal brain are calcium dependent. NOS in the brain is inactive at resting free Ca^{2+} concentrations, but becomes fully active when the Ca^{2+} concentration increases. Since eNOS and nNOS are both Ca^{2+} dependent, either of these could be activated in Knowles et al.'s experiments. The form of NOS that is typically found in astrocytes is iNOS which is over 90% identical to other forms of iNOS found in mouse macrophages, rat hepatocytes, and rat smooth muscle [60]. The inducible form of NOS is calcium-independent [60, 104]. During pathological situations evidence suggests that glial cells are also capable of expressing calcium-dependent eNOS [89, 104]. Similarly there are two types of NOS isoforms found in C6 cells, eNOS that is active in pathological situations and iNOS [16, 49, 104].

Nitric oxide is functionally important during immune responses in glial cells, including C6 cells. Superoxide release from C6 cells stimulates NO formation via NOS. When SOD increases, changing superoxide to hydrogen peroxide, the NO levels in the cell decrease [104]. Barna et al. found that there is an increase in the eNOS expression in astrocytes when neurons are infected with vesicular stomatitis virus (VSV) [16]. Astrocytes thus increase their production of NO, killing the virus so that the virus does

not infect surrounding cells. It is also possible for NO to diffuse out of the cell to neighboring infected cells in order to kill the virus. They also found that the neurons, not the astrocytes, were infected with the virus when astrocytes increased their NO production. The neurons were signaling the astrocytes to increase eNOS expression for NO production. The NO then diffused into the neurons to kill the virus [16].

Other ROS Producers

While NADPH oxidase, mitochondrial ETC, xanthine oxidase, and NOS are responsible for most of the ROS production in cells, other sources of ROS include oxidation of dopamine [43] and activity of peroxidases [62].

Cell Signaling

ROS are able to act as intracellular signals because they can diffuse across short distances, are easily synthesized, and easily degraded [59, 92]. Many were skeptical of ROS acting as an intracellular signal because their high reactivity cause them to interact with the first organic molecule to which they come in contact [129]. However, we now know that ROS can activate pathways which lead to the phosphorylation of molecules such as protein kinases, protein phosphatases, phospholipases, ion channels, and small GTPases [129].

Transcription Factors

Transcription factors are important gene regulators within the cell that are used to control the rate of transcription of specific genes. When under physiological stress, such

as viral or bacterial attacks, cells must produce certain proteins to fight these attacks. ROS act as intracellular signals in these conditions to activate transcription factors via oxidation of cysteine residues. The oxidation of sulphydryl groups forms disulphide bonds and results in intra or inter-molecular bonds [37].

Two transcription factors known to be activated by ROS are nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) [144]. NF-κB activates genes critical for early cellular defense reactions. Similarly, AP-1 is activated to regulate stress response genes that control cellular proliferation and apoptosis [59]. NF-κB is typically found in the cytoplasm of a cell where it is inactivated by binding IκB [62]. Activation of NF-κB is accomplished by phosphorylating IκB which releases it from NF-κB [50, 59, 62, 65, 144]. Then NF-κB is active and able to move to the nucleus to begin transcription [62]. The ROS molecule, H_2O_2 is an effective activator of NF-κB [59, 144]. The most probable way in which H_2O_2 activates NF-κB is through activation of a kinase that then phosphorylates IκB [59].

AP-1 is made up of a homodimer, Jun, and a heterodimer, Fos. Both dimers are present within the cell, but dormant until phosphorylated. Phosphorylation of AP-1 causes the two dimers, Jun and Fos, to come together. This provides a binding site for the DNA sequence containing the appropriate AP-1 sites and thus initiates transcription. Due to the presence of two sites on the complex that are phosphorylated by interaction with ROS, activation of AP-1 is highly dose dependent. Phosphorylating AP-1 a second time greatly enhances its activity. Similar to NF-κB, the redox state within the cell affects the phosphorylation status of AP-1. During oxidative stress AP-1 is phosphorylated at both sites on the complex which causes greater activation of AP-1

during these conditions [59, 144]. Many cells use the redox state to activate both AP-1 and NF-κB including astrocytes and more specifically C6 cells [75, 85, 95, 99, 109].

Another role of NF-κB and AP-1 in astrocytes and C6 cells is the production of NO. NF-κB and AP-1 initiate NO production by activating iNOS. Synthesis of NO is important in immune response by acting as an antitumor agent [85, 95].

Ion Channels

ROS also function as ion channel regulators. They can do this in several ways: direct modification of the channel proteins to an active state, activating other signaling mechanisms that in turn activate the channel, or changing gene expression of the channel proteins [110].

In astrocytes, H_2O_2 activates channels that increase intracellular Ca^{2+} [6, 72]. Through this mechanism, hydrogen peroxide may modify physiological functions such as secretion, synaptic plasticity, gene expression, muscle contraction, channel activation, cell proliferation, and cell death [6, 72, 152]. For example, H_2O_2 acts by inducing phosphorylation of PLCγ1. This then activates subsequent messengers which increase intracellular calcium levels and activate protein kinase C [72].

Apoptosis

Apoptosis or programmed cell death is a type of cell death that is controlled by mechanisms within the cell. It can be used to remove unwanted cells from tissue without disrupting the surrounding tissue [143]. This form of cell death, which has been found in many different cell types [25, 93, 161], occurs in two defined stages. The first stage of

apoptosis is characterized by nuclear and cytoplasmic condensation. Thus, the cytoplasm shrinks, as does the nucleus, eventually breaking up into small membrane-bound fragments known as apoptotic bodies. Another characteristic of apoptotic cell death is the externalization of phosphatidylserine. Phosphatidylserine is only found on the inside of the cell membrane, but as cells become apoptotic they flip the phosphatidylserine to the outside of the membrane. Experimenters have used this as an early indicator of apoptosis by using a fluorescent dye, annexin V, to bind to the external phosphatidylserine molecules [134]. The second stage of apoptosis occurs when apoptotic bodies are phagocytosed by neighboring cells [143]. Some of the triggers for apoptosis are hormones or growth factors during development, organismal protection through the immune system by killing off viral or bacterial infected cells, or in response to cell injury [143]. Apoptosis uses ATP to control characteristic events such as cell shrinkage, apoptotic volume decrease, nuclear condensation, DNA, RNA, and protein cleavage by endonucleases, mitochondrial depolarization, and eventual blebbing off the cell membrane, forming an apoptotic body [17, 25, 26, 93, 97, 123].

Apoptosis can be initiated by intercellular or intracellular signals. Intercellular signaling of apoptosis occurs via transmembrane receptors called death receptors. These receptors bind to specific signals, death signals, such as tumor necrosis factor that induce the apoptotic pathway. When the death signals bind to the extracellular portion of the receptor it induces activation of caspases 8 and 9. These 2 caspases activate the cascade of events that will end in apoptotic cell death.

Intercellular signaling of apoptosis causes the release of cytochrome c from mitochondria and a protein factor called Apaf-1. These signals activate caspase 9

initiating the apoptotic cascade [10]. Some intracellular signals known to play a role in inducing apoptosis include an increase in intracellular calcium, activation of protein kinases A and C, and alterations in gene expression [143]. The presence of ROS can increase the probability that the cell will become apoptotic. This action of ROS may be mediated by ERK, p53, NF-κB, and caspase-11 [87, 96, 157, 160].

Extracellular signal-regulated kinase (ERK) plays a role in regulating cell growth, survival, and differentiation. Lee et al. found H_2O_2 increased the activation of ERK leading to apoptotic cell death; however, the mechanism of ERK induced apoptosis is somewhat unclear. H_2O_2 leads to the phosphorylation of ERK using a growth factor receptor and Ras signaling pathway [96].

ROS also induces the accumulation of p53. During the cell cycle p53 is a critical regulator at the G1 checkpoint. p53 stops cells that have DNA damage from entering mitosis and induces apoptosis of the cell if the damage is irreparable. Cells exposed to H_2O_2 have increased DNA fragmentation and chromatin condensation, similar to that observed in apoptosis, and an increase in p53 expression. Thus, cells damaged by oxidative damage tend to enter apoptosis if p53 cannot activate the repair of the DNA [87].

An important activator for apoptosis in astrocytes is a rapid increase of intracellular calcium. Calcium can accumulate in the cell if the Na^+-Ca^{2+} exchanger is reversed. This process then induces apoptosis [143, 159, 160]. Most studies show a need for intracellular Ca^{2+} increase to activate apoptosis [143, 160].

Apoptosis during inflammatory responses in C6 cells is controlled by two parallel pathways. One is a signaling cascade using caspase 11 and the other is a direct

stimulation of apoptosis with ROS. Caspase 1, 3, and 11 are major inducers of apoptosis [35, 157]. It has been shown that caspase 11 is crucial for the induction of apoptosis and that it is caspase 11 that activates the down stream caspases 1 and 3 [157]. In C6 cells, increased ERK phosphorylation and increased expression of p53 during H_2O_2 exposure suggests that ROS plays a similar role in apoptosis for C6 cells compared with other glial cell lines [35, 157].

Immune Response

When phagocytic cells such as neutrophils, eosinophils, and macrophages are exposed to invading microorganisms they exhibit a series of fast responses which include cytoskeletal rearrangement, motility, exocytosis of secretory granules, and activation of NADPH oxidase. NADPH oxidase is activated to rapidly produce ROS as a means of killing bacteria and viruses [56]. The increase in ROS is very harmful to the invading microorganisms killing them off rapidly, but it is also harmful to the host cells. ROS in high doses can cause damage to cells such as lipid peroxidation, protein disruptions, and DNA splicing. Neutrophils and eosinophils produce a higher concentration of ROS than macrophages which accounts for the greater longevity of macrophages [57].

A respiratory burst is stimulated in a macrophage by depolarization of the membrane and an increase in intracellular free calcium. The cell then activates the NADPH oxidase complex to produce ROS within the confines of an organelle, the phagosome. The respiratory burst is transient. Calcium plays a role in both initiating and terminating the respiratory burst; however, the exact mechanism of calcium is still unclear. When initiating the respiratory burst, calcium is needed to activate $p47^{phox}$

translocation to the membrane allowing the assembly of NADPH oxidase complex. Buffering intracellular calcium levels eliminates both enhancement and termination of the respiratory burst and shows the vital role that calcium plays [56, 57, 73, 117].

Other Roles of ROS

Studies have determined that ROS are used to regulate intracellular pathways involved with growth, cell division, transformation, apoptosis, and senescence [52]. For example, protein tyrosine phosphatase has a cysteine residue that can interact with H_2O_2 causing activation [51]. Once activated, tyrosine phosphatase is responsible for the control of normal growth and proliferation of the cell [51, 54].

Cell Injury

While cells are able to use low concentrations of ROS as intracellular signals, high concentrations of ROS cause cellular damage. The level of ROS within a cell is critical for cell survival and thus is highly regulated via rates of ROS production and the level of antioxidants. If the concentration of ROS increases above the level which the antioxidants can control, the cell becomes apoptotic, necrotic, or neoplastic [108]. Many diseases also can occur if the redox state within the cell is not at physiological levels such as Alzheimer's disease, Parkinson's disease, diabetes insipidus, diabetes mellitus, Huntington's disease, and amyotrophic lateral sclerosis [6, 66, 115, 140]. The key ways in which uncontrolled ROS causes harm to the cell are protein peroxidation, lipid peroxidation, and damage to DNA.

Lipid Peroxidation

The chief components of cell membranes are polyunsaturated fatty acids [18]. Polyunsaturated fatty acids are oxidized in a normal biological process known as lipid peroxidation [36]. But like many other processes, lipid peroxidation must be tightly controlled within the cell. Uncontrolled lipid peroxidation causes a loss of membrane integrity and increased membrane fluidity. In these conditions the cell is at a higher risk of cell death [11, 36, 67].

The general mechanism for lipid peroxidation occurs in three steps, initiation, propagation, and termination. The first step is initiation during which free radicals are formed. The initiation step of lipid peroxidation occurs when a lipid reacts with a free radical. Polyunsaturated fatty acids have at least one, and usually more than one, methylene groups between cis double bonds [107]. The hydrogen atoms on the methylene groups are readily removed to form carbon-centered free radicals which react with molecular oxygen to form peroxyradicals.

The second step of lipid peroxidation is the propagation step. This is when the oxygen free radical formed in the initiation step reacts with other lipids to form more free radicals. This reaction produces another carbon centered radical which then can further propagate the reaction. Each radical initiated can lead to many propagation steps.

The third step in lipid peroxidation is termination. Termination of lipid peroxidation only occurs when a free radical interacts with an antioxidant like vitamin E or combines with another free radical. When two free radicals combine they share their unpaired electrons to form a molecule that is not a free radical [42, 107, 131]. Antioxidants reduce peroxyl radicals to hydroperoxides breaking the reaction chain and

slowing the rate of lipid peroxidation. The concentration of vitamin E in the lipid bilayer is therefore very important to control the amount of lipid peroxidation that occurs [130].

Neurons and glia are especially susceptible to oxidative damage because of the large amounts of oxygen consumption, high concentrations of transition metal ions and low concentration of antioxidants in the brain [106]. Another reason the brain is vulnerable to oxidative damage is that it has an abundance of polyunsaturated fatty acids which are the primary target for lipid peroxidation [105].

DNA Damage

Oxidation of DNA strands causes the strands to break, DNA-DNA crosslinking, direct oxidation of base pairs, DNA-protein crossbridges, and base modifications [30, 106]. This can lead to altered transcription and translation levels within the cell, and eventual cell death. Mitochondrial DNA are more susceptible to DNA damage than nuclear DNA because there are more ROS in the mitochondria [53, 106]. Histones and DNA repair mechanisms, not present in mitochondria, also protect nuclear DNA [106]. Once the mitochondrial DNA is damaged, the mitochondria produce more ROS causing a chain reaction of DNA damage and ROS production [53]. DNA damage is not only caused by ROS but also by lipid peroxidation. The peroxy radicals formed during lipid peroxidation react with DNA and break DNA strands similar to the actions of ROS [101].

In C6 cells, increased H_2O_2 production causes DNA fragmentation [166]. When the membrane integrity is changed due to lipid peroxidation, free radicals are more able to enter the nucleus and attack chromatin. The metal ions within the cell will also play a

role in changing H_2O_2 to hydroxy radicals in or around the chromatin. Hydroxy radicals are able to nick a single strand of DNA which can then lead to double stranded breaks. The nicks usually occur at GG and GGG sequences within the DNA [67, 139].

Aging

Aging is a normal part of the life of an organism. ROS is known to play a role in normal aging, and in high concentrations it also can accelerate the aging process. The molecular theory of aging states that DNA damage and DNA errors decrease lifespan [168]. An early sign of aging and neurodegeneration is an increase in ROS production above the normal range to levels that are toxic to the cell [41]. The change in the redox state within the cell alters neuronal excitability and ion homeostasis [11, 41]. These changes in redox potential account for some of the changes that cause normal aging and neuodegeneration, but they do not explain all of the neuronal dysfunction [41].

A disease state of a cell occurs when the intracellular redox potential is no longer inside physiological ranges. ROS decreases lifespan by causing biological damage and degenerative diseases associated with aging such as cancer, cardiovascular disease, immune system dysfunction, decline in brain function, and cataracts [9, 147]. The oxidative shift which results from excessive ROS production can produce a toxic environment within the cell and can cause DNA damage, lipid peroxidation, and telomerase shortening resulting in senescence of the cell [9, 41, 148, 164]. When ROS causes shortening of the telomerase it signals the cell to exit proliferation and remain in the G1 phase. The rate of telomerase shortening is increased in cells that are exposed to

mild hyperoxic environments where intracellular ROS is increased [164]. This suggests that a shift in the redox potential in cells will result in the cell entering senescence early.

DNA damage is normally repaired immediately or cell division ceases, but not all of the lesions are repaired [3, 9]. As an organism ages the amount of unrepaired DNA lesions and mutations increase. According to the molecular theory of aging this increase in damaged DNA leads to the shortening of the lifespan of the organism [3, 9].

Summary of Study

Cells swollen by exposure to hypoosmotic conditions must regulate their volume for survival. The activation of RVD mechanisms are not completely understood. Mechanisms that seem to play a role in the activation of RVD are ROS production and activation of extracellular purinergic receptor by extracellular ATP. ROS produced during hypoosmotic swelling and/or the purinergic receptor activation then activate VSOR Cl^- channels and allows the cells to begin RVD. Overproduction of ROS can cause cell death either by apoptosis or necrosis. Thus in order for the cell to benefit from the ROS production the cell must tightly control how much ROS is produced. Many studies have determined that ROS acts as an intracellular signal in a gamut of cell types. This study was designed to determine if ROS was produced during hypoosmotic exposure in a rat gliomal cell line (C6 cells) and if that ROS was produced at a high enough concentration to cause cellular injury. I also explored interaction between ROS production and ATP release during hyposmotic exposure.

II. Specific Aims:

Ionic gradients across the plasma membrane are critical for proper cell function. When cells are exposed to hypoosmotic conditions and excess water moves into the cell, the resulting alteration of ion concentrations within and around the cell can have detrimental effects. To recover from osmotically induced changes in cell size, volume regulatory mechanisms are initiated in response to signals which may include the release of ATP and an increase in the rate of production of reactive oxygen species (ROS). In considerable amounts, ROS also may cause injury and cell death. Thus, I hypothesize that when cells swell due to exposure to hypoosmotic conditions a resulting increase in ROS production leads to cytotoxicity and death. I will directly test this hypothesis by examining mechanisms of hypoosmotic-induced ROS production in rat glioma C6 cells and determine if swelling-induced oxidative stress leads to cellular injury. I also will examine the role that extracellular ATP plays for increased ROS production.

Specific Aim I

I hypothesize that C6 cells swollen in hypoosmotic conditions increase their production of ROS via activation of NADPH oxidase. While swelling the cells in hypoosmotic conditions, I will measure the rate of intracellular ROS production using a spectrofluorometer and ROS-sensitive dyes. Some experiments will be carried out in the presence of pharmacological agents to block specific producers of ROS including NADPH oxidase and mitochondria electron transport chain.

Specific Aim II

I hypothesize that C6 cells swollen in hypoosmotic conditions increase ROS production via activation of extracellular purinergic receptors. I will expose the C6 cells to hypoosmotic conditions and measure the release of ATP. I also will examine the effect of ATP exposure on ROS production in isoosmotic and hypoosmotic conditions.

Specific Aim III

I hypothesize that swelling-induced increased ROS production increases the probability of cell death. I will determine the magnitude of cell death by measuring release of lactate dehydrogenase and with cytochemical markers of cell injury and death.

These studies will examine the relationship between the ROS production and volume regulation. It will further our understanding of the regulation of the NADPH oxidase and mitochondrial ROS generating complexes in glial cells and their potential role in the regulation of cell volume and production of cell injury. This information will provide insight into cell signaling process of cells exposed to hypoosmotic conditions.

III. Materials and Methods:

Materials

5-(and-6-)-dichloroddihydrofluorescein diacetate (DCFDA), and annexin V came from Molecular Probes (Eugene, OR). Trypsin, trypan blue, horse serum, fetal bovine serum, penicillin, and streptomycin came from InVitrogen (Grand Island, NY). Trolox C came from Aldrich Chemical Company (Milwaukee, WI). All other chemicals including sodium bicarbonate, poly-L-lysine hydrochloride, carbenoxolone, dibenziodolium chloride (DPI), ascorbic acid, rotenone, oligomycin, *in vitro* lactate dehydrogenase based toxicity assay kit (LDH kit), bicinchoninic acid kit for protein determination, ATP, and hydrogen peroxide came from Sigma-Aldrich (St. Louis, MO) or Fischer Scientific (Hanover Park, IL).

Glial Cultures

Frozen C6 cells in suspension were obtained from American Type Culture Collection (Manassas, VA). We quickly thawed the cells to 37 ºC and plated them onto a 25 cm^2 flask containing 3 ml of growth medium consisting of Ham's F-12 Kaign's medium (Ham's F-12K) containing 2 mM L-glutamine (Sigma-Aldrich, St. Louis Missouri) plus 15% horse serum, 2.5% fetal bovine serum, 50 units/ml penicillin, and 50 µg/ml streptomycin. Ham's F-12K medium was made from powder and was supplemented with 1.5 g/L sodium bicarbonate according to the manufacturer's directions. We first prepared aliquots of cells for freezing so that additional C6 cells would be available for later study. Cells were grown until confluent and then were removed from the dish and replated onto 10, 25 cm^2 flasks. To remove the cells from the

flask for replating, we first replaced the growth medium with 0.25% trypsin in Hank's balanced salt solution. When the cells had been loosened from the culture surface (5 - 15 min) we added complete medium to inactivate the trypsin. The resulting cell suspension was centrifuged for 5 min at approximately 300 x g and the supernatant medium with trypsin was decanted and replaced with fresh growth medium for plating onto new culture flasks. After these cell cultures had grown to confluence, the cells were removed with trypsin as described above, centrifuged, and resuspended in complete growth medium (1 ml per culture flask) containing 5% dimethylsulfoxide as a cryoprotectant. The suspension was divided into Nalgene cryogenic vials (Rochester, NY), slowly cooled in stages to 4 ºC, -20 ºC and -70 ºC and then placed in liquid nitrogen for long-term storage.

C6 cells were replated weekly to maintain cells below confluence. Cells were plated at different densities so that experiments performed on different days of the week used cultures of similar cell density (approximately 75% of confluence). Cells were placed in 24-well plates or in 35 mm dishes. Some of the 35 mm dishes contained glass coverslips. The growth surfaces were coated with poly-L-lysine hydrochloride prior to plating the cells by treating the surfaces for 120 min with a solution of 0.05 mg/ml poly-lysine in sterile water. Cells were maintained at 37 ºC in an atmosphere of room air with 5% CO_2. The growth medium was changed to serum-free media Ham's F-12K plus 50 units/ml penicillin, and 50 μg/ml streptomycin 24 hours prior to experimentation.

Reactive Oxygen Production

To evaluate the production of reactive oxygen species (ROS) by C6 cells, we measured the fluorescence of an exogenous intracellular fluorescent dye using a computer-controlled SLM 8000C spectrofluorometer. Cells were first incubated for 15 min with 50 μM of DCFDA in a phosphate-buffered saline (PBS) solution at 37 ºC. PBS consisted of 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, and 5.5 mM glucose (290 mOsm). Osmolality was adjusted using small volumes of 3.0 M NaCl and confirmed using vapor pressure osmometry. After loading the cells we moved the coverslip to the fluorescence cuvette in the spectrofluorometer. The coverslip was placed in a Teflon coverslip holder in a standard fluorescence cuvette (1.2 cm × 1.2 cm × 4.5 cm), and held at about 30º relative to the excitation light path. The perfusion solution was placed into a 20 ml syringe and delivered to the cuvette at 1 ml/min using a programmed syringe pump. The syringes containing perfusion solution and the chamber of the spectrofluorometer holding the cuvette were warmed in a water bath set at a temperature which maintained the cells at 37 ± 1 ºC. A stainless steel tube delivered the perfusion solution to the bottom of the cuvette under a baffle to reduce turbulence. Thus, the solution filled the cuvette from the bottom and flowed upwards over the cells. As the perfusion solution pooled at the top of the chamber, it was removed by continuous suction via a second stainless steel tube positioned above a second flow baffle at the top of the cuvette [132].

The cells typically were held in the chamber for 20 min. During the first 10 min cells were perfused with isoosmotic PBS. Then I changed the perfusion solution to an experimental PBS solution for the next 10 min. Experimental solutions were made

hypoosmotic (200 mOsm or 150 mOsm) by reducing the concentration of NaCl. Pharmacological agents such as trolox C (150 μM), ascorbate (400 μM), DPI (10 μM), rotenone (5 μM), or oligomycin (10 μM) also were added to some of the experimental PBS solutions. When an agent was used in the hypoosmotic PBS, it also was added to isoosmotic PBS for 5 min prior to hypoosmotic exposure.

Evaluation of Cell Death

Cell death was evaluated using trypan blue, to label necrotic cells, or annexin V, to label apoptotic cells. DAPI was used to stain nuclei of all the cells. Serum free growth medium was removed from the dishes and the cells were rinsed two times with sterile isoosmotic PBS. Then the sterile experimental PBS was added to the dishes and the cells incubated for one hour at 37 °C. I rinsed the dishes again with sterile isoosmotic PBS, fed the cells with fresh serum-free growth medium, and returned the dishes to the incubator. After 24-hours the cells were taken from the incubator and medium was removed. Isoosmotic PBS was added to the cells for 5 min. Then the solutions were changed to a 2% trypan blue solution in Hanks salt solution or annexin V diluted 1:1000 in isoosmotic PBS for 10 min. This solution was removed and the cells rinsed with PBS before exposure to 70% ethyl alcohol for 10 min. Ethanol was removed and DAPI stain was added to the cells at a concentration of 0.5 μg/ml in isoosmotic PBS for 10 min. Cells then were rinsed for 5 min in isoosmotic PBS and then 5 min in distilled water. Coverslips were then fixed to glass slides and allowed to dry for 24 hours.

The release of lactate dehydrogenase (LDH) into the growth medium was used as a measure of cell death via necrosis following hypoosmotic exposure. LDH was

determined in growth medium and attached cells using a commercial kit (Sigma-Adrich, St. Louis, MO). Serum free growth medium was removed from the dishes and the cells were rinsed two times with sterile isoosmotic PBS. Then the sterile experimental PBS was added to the dishes and the cells incubated for one hour at 37 °C. The dishes were rinsed again with sterile isoosmotic PBS, fed with fresh serum-free growth medium, and returned to the incubator. The serum-free growth medium was removed 24 hr later and saved for LDH analysis. The cells then were scraped from the dishes into 1 mL of fresh serum-free growth medium. This solution was sonicated 3 times for 5 s. LDH was measured after diluting 5 μL of the sonicated cell solution with 295 μL of fresh serum-free growth medium or 150 μL of the growth medium with 150 μL of fresh serum-free growth medium. Then 600 μL of LDH assay solution was added to each diluted sample following the directions of the assay kit. This solution was incubated in the dark for 30 min at room temperature. After this time I measured the absorbance at 490 nm using a spectrophotometer.

ATP release

The release of ATP from cells during exposure to isoosmotic and hypoosmotic PBS solutions was determined using a commercially available kit based on luciferin luminescence. We designed these experiments to mimic the solution changes used for the fluorescence studies. Cells were rinsed twice with isoosmotic PBS and then were incubated for a total of 20 minutes in isoosmotic PBS prior to exposing the cells to the experimental solution. When pharmacological agents were present in the experimental solution, they also were added to the isoosmotic PBS for the last 5 min, similar to the

procedure used for spectrofluorimetric analyses. Some solutions also contained carbenoxolone. After treatmen, the PBS solution was removed from the cells and stored in an ice bath until analyzed for ATP content. Cells were dissolved from the culture well for protein measurements using 0.5 ml of 1 M sodium hydroxide for 20 min.

To measure ATP, contemporaneous standards were prepared using a 1 mM stock solution of ATP. The amount of ATP in each sample was determined by preparing 5 standards with different amounts of ATP (0 pmols, 0.04 pmols, 0.12 pmols, 0.21 pmols, and 0.42 pmols). The luciferin assay was prepared by combining a 1:9 ratio of ATP assay mixture and ATP buffer from the kit. Each sample tube received 100 μL of the diluted assay mixture for 3 min of incubation to remove endogenous ATP in the solutions. Then 100 μL of sample or standard was added and luminescence was measured immediately using a 1250 Luminometer (LKB Finland). Solutions were placed in the luminometer chamber and the measurement was taken once reading the settled at a peak height.

Protein was measured using a bivinchoninic acid kit for protein determination according to the manufacturer's directions. The protein content of the standards was 0 ug, 10 μg, 20 μg, 30 μg, 40 μg and 50 μg. Absorbance of each sample and standard was determined at the suggested wavelength (562 nm) in a spectrophotometer.

Summary of Statistics:

Fluorescence data was analyzed by repeated-measures ANOVA. Comparisons of LDH release between experimental groups were performed using Student's t test or Z

test. Counts of apoptotic or necrotic cells were compared with chi square analysis. Significance was indicated for $P < .05$

IV. Results

Oxidized DCF Excitation Spectra

DCFDA freely diffuses into the cell where the two acetate groups are removed by endogenous esterases, forming 5-(and-6-)-carboxy-2,7-dichlorodihydrofluorescein (DCF) [52]. The removal of the acetate groups leaves the dye charged at physiological pH and thus, unable to move freely out of the cell through the cell membrane. In this form, DCF is weakly fluorescent; however, when it reacts with a ROS molecule it is oxidized and its fluorescence efficiency increases significantly. Data from the manufacturer indicate the excitation and emission wavelengths for maximum fluorescence of oxidized DCF (oxDCF) are 488 nm and 529 nm, respectively. To determine if the oxDCF excitation spectrum has a similar peak when the dye is within C6 cells I ran a spectrum using a 529 nm emission wavelength for excitation wavelengths ranging between 400 and 520 nm (Figure 1). Cells were loaded with DCFDA for 15 min and then moved to the fluorescence perfusion chamber as described in Methods. The sharp peak seen in Figure 1 at 469 nm is similar to the excitation peak for oxDCF in the literature [1]. The peak is sitting on top of a broad non-specific endogenous fluorescence signal from the cells.

Oxidized DCF Emission Spectra

The emission spectrum in Figure 2 obtained from oxDCF in C6 cells looks similar to the emission spectrum found in the literature [12]. The emission peaks for excitation wavelengths of 475 nm, 480 nm, and 485 nm were 527, 525, and 524, respectively. The

Figure 1: Excitation spectrum of oxidized DCF in C6 cells. C6 cells were loaded with DCF for 15 min and then were transferred to a fluorescence cuvette for perfusion with 37 ºC isoosmotic PBS. The excitation spectrum over the range 400 nm to 520 nm was obtained at an emission wavelength of 529 nm. The bandwidth of the excitation and emission monochrometers (FWHM) was 8 nm.

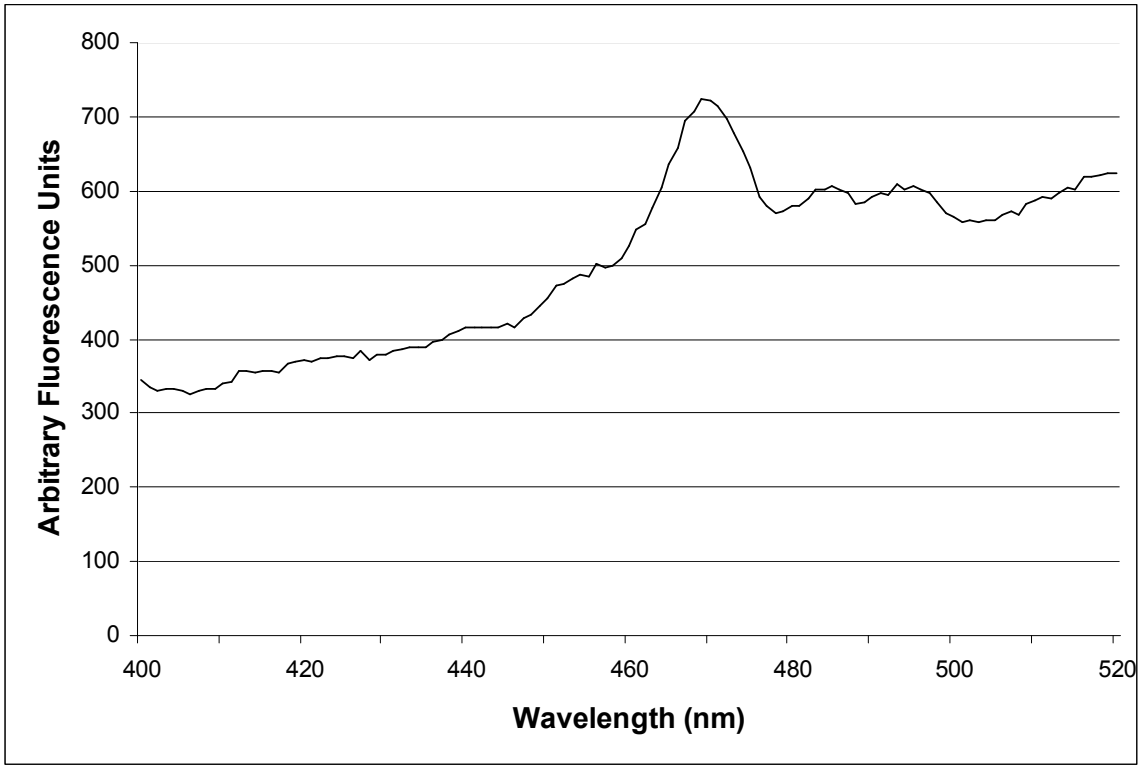

800
700
600
500
400
300
200
100
0
Arbitrary Fluorescence Units
400
420
440
460
480
500
520
Wavelength (nm)

Figure 1

Figure 2: Emission spectra of oxidized DCF in C6 cells. C6 cells were loaded with DCF for 15 min and then were transferred to a fluorescence cuvette for perfusion with 37 ºC isoosmotic PBS. Fluorescence intensity between 500 nm and 600 nm was measured for different excitation wavelengths as indicated. Scattered excitation light can be seen in each spectrum from 500 nm to about 510 nm. The peak of the Raman water signal can be seen between 560 nm and 585 nm (within box).

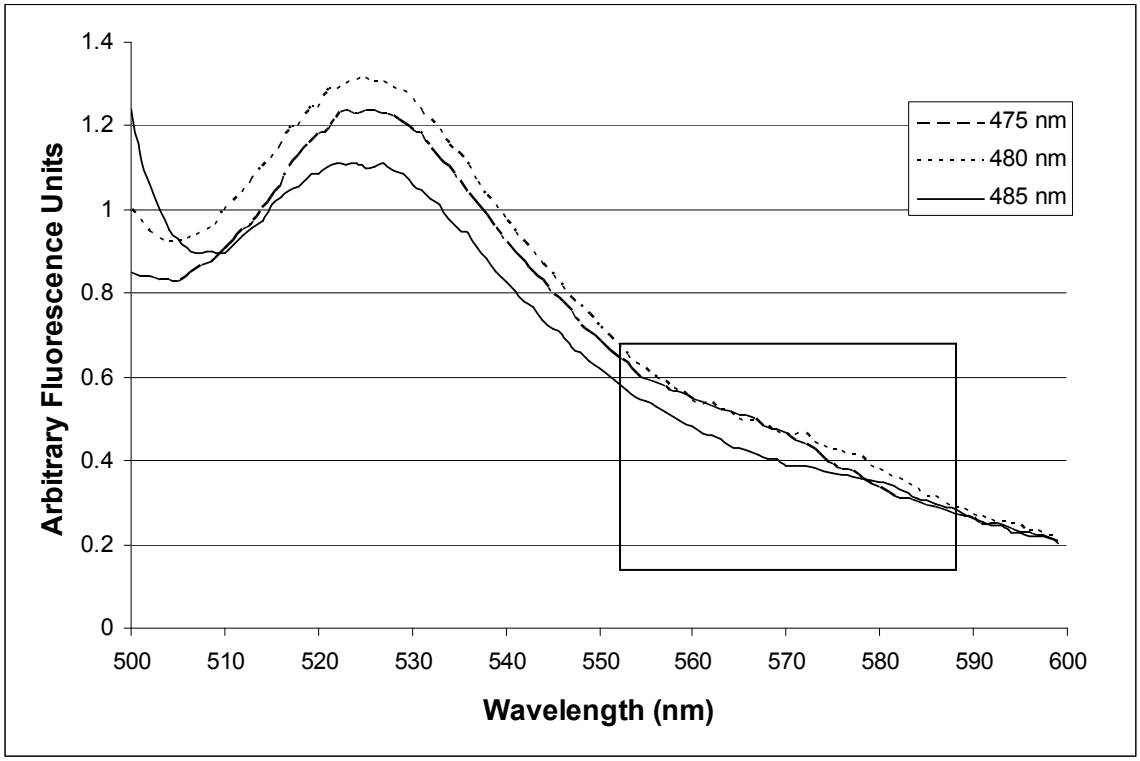

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Arbitrary Fluorescence Units
500
510
520
530
540
550
560
570
580
590
600
Wavelength (nm)
475 nm
480 nm
485 nm

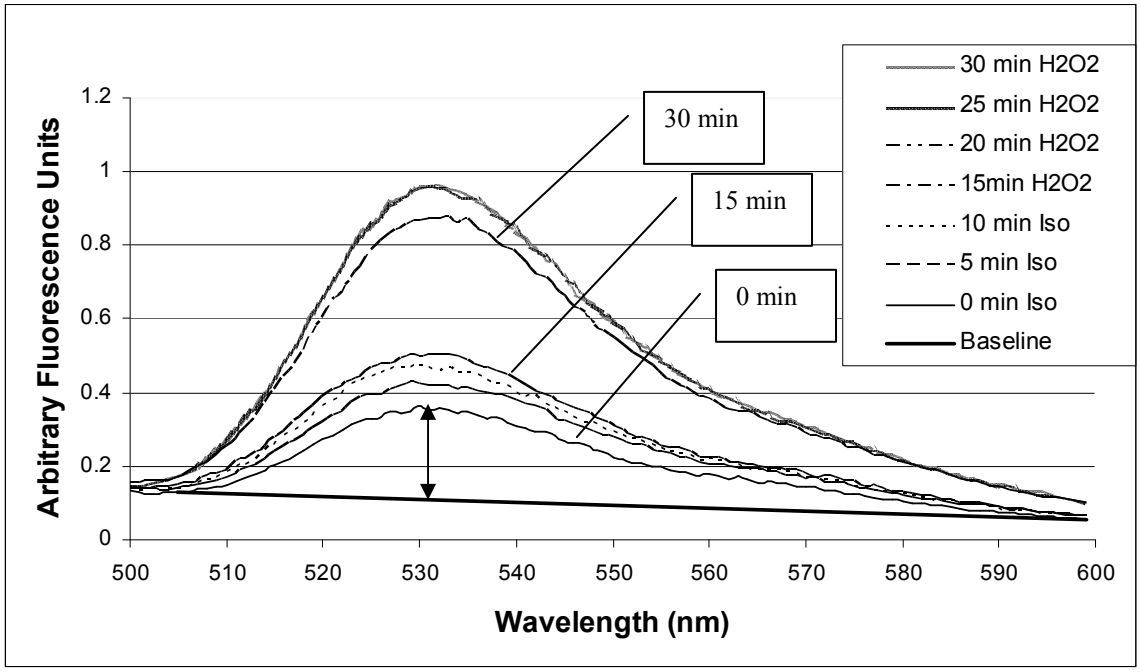
Figure 2

excitation wavelength was first set at 475 nm (8 nm FWHM) and an emission spectrum was measured from 500 nm to 600 nm. The resulting spectrum of oxDCF appeared to rest on a baseline whose magnitude decreased with increasing wavelength (Figure 2). Figure 2 shows a rapid drop off in arbitrary fluorescence units from 500 nm – 510 nm. The fluorescence signal in this region is most likely from scattered light from the excitation beam. Because the concentration of oxDCF is expected to be very low during the initial period of fluorescence measurement I evaluated potential interference of Raman fluorescence with the oxidized DCF fluorescence signal in the C6 cells. The Raman signal for water is made up of several broad peaks centered at 563 nm for an excitation of 470 nm and centered at 577 nm for an excitation of 480 nm [31]. Raman fluorescence can be seen as broad peaks (Figure 2). When the excitation wavelength was moved from 475 nm to 485 nm peak, the Raman spectrum moved from 570 nm to 580 nm on the long wave length tail of the oxDCF spectrum. Moving the excitation wavelength decreased the interference of the Raman spectrum with the oxDCF signal.

*ROS Production***:**

Initial studies of ROS production were measured with the cells perfused in isoosmotic PBS (Figure 3). Emission spectra were taken every 5 min for 30 min while perfusing the cells with isosmotic PBS as described in Methods. The baseline underlying the oxDCF spectrum was assumed to be a straight line between 500 and 600 nm (solid black line, Figure 3). To correct for this baseline when measuring oxDCF fluorescence, I measured the emission at three wavelengths; 505 nm, 525 nm, and 600 nm. The peak

Figure 3: Progressive oxidation of DCF in C6 cells. C6 cells were loaded with DCF for 15 min and then were transferred to a fluorescence cuvette for perfusion with 37 ºC isoosmotic PBS. Emission spectra were acquired every 5 min using an excitation wavelength of 475 nm. After 15 min 200 μM H_2O_2 was added to the isoosmotic PBS. The baseline is a straight line drawn between the fluorescence intensity values measured at 600 nm and 505 nm for the 0 min spectrum.


30 min H2O2
25 min H2O2
20 min H2O2
15min H2O2
10 min Iso
5 min Iso
0 min Iso
Baseline
30 min
15 min
0 min
Arbitrary Fluorescence Units
Wavelength (nm)
1.2
1
0.8
0.6
0.4
0.2
0
500
510
520
530
540
550
560
570
580
590
600

height of the emission spectrum at 525 nm then was calculated relative to a linear baseline calculated from the fluorescence emission signal measured at 505 and 600 nm.

The level of oxDCF within the cell increased in a time-dependent manner for 15 min, indicating a steady production of ROS. After 15 min I added 200 μM H_2O_2 to the perfusing PBS and continued to acquire spectra for an additional 15 min. There was a 2-fold increase in the quantity of oxDCF within 5 min of introducing H_2O_2 to the perfusing PBS. After this initial burst of ROS production, the rate of increase in oxidized dye leveled off suggesting that the H_2O_2 oxidized all the available unoxidized dye.

In initial studies, some of the cover slips showed unstable dye fluorescence during solution changes. When solutions were changed there often was a sudden drop off in the fluorescence intensity followed by an increase after 1-2 min in the new solution (Figure 4a). This drop in fluorescence intensity could be due to mechanical stress on the membrane from the flow of PBS. In order to decrease the mechanical stress and help the cells to stay on the coverslip better, I coated the coverslips with poly-lysine and decreased the flow rate from 3 ml/min to 1 ml/min (data not shown). The drop in fluorescence intensity did decrease with the slower perfusion rate, but the drop in intensity was still consistently present. A possible cause for the drop in fluorescence was efflux of the dye through hemi channels or other membrane channels in the cell membrane that were activated during cell swelling. To block dye efflux through the pathways I added 250 μM sulfinpyrazone or 100 μM carbenoxolone to all perfusing solutions. Sulfinpyrazone had no effect on the apparent dye efflux (data not shown).

Figure 4: DCF efflux is prevented with carbenoxolone. C6 cells were loaded with DCFDA for 15 min and then were transferred to a fluorescence cuvette for perfusion with 37 ºC isoosmotic PBS. The fluorescence from oxDCF was measured while cells were perfused with isoosmotic PBS for 10 min. Then the perfusate was changed to hypoosmotic PBS for an additional 10 min A. DCF fluorescence in C6 cells during perfusion with isoosmotic PBS, hypoosmotic PBS, and isoosmotic PBS containing 0.2 mM H_2O_2. B. DCF fluorescence in C6 cells during perfusion with isoosmotic and hypoosmotic PBS containing 100 μM carbenoxolone.

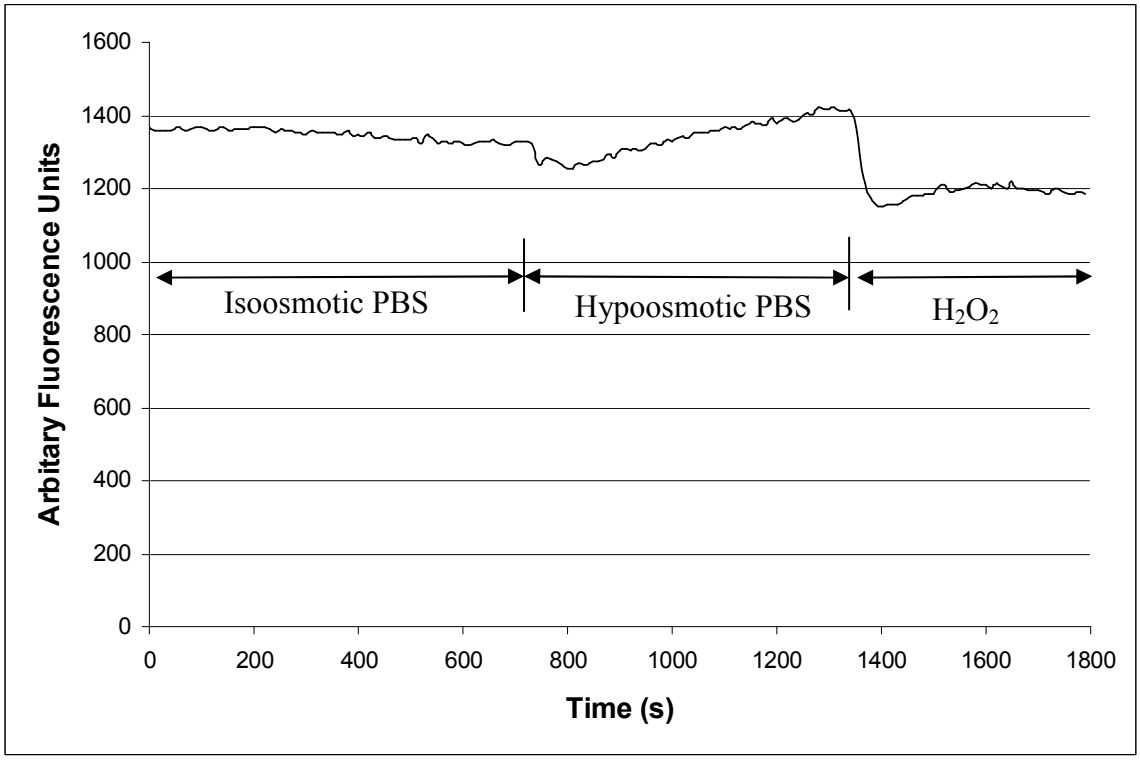

Arbitary Fluorescence Units
1600
1400
1200
1000
800
600
400
200
0
0
200
400
600
800
1000
1200
1400
1600
1800
Time (s)
Isoosmotic PBS
Hypoosmotic PBS
H2O2

Figure 4a

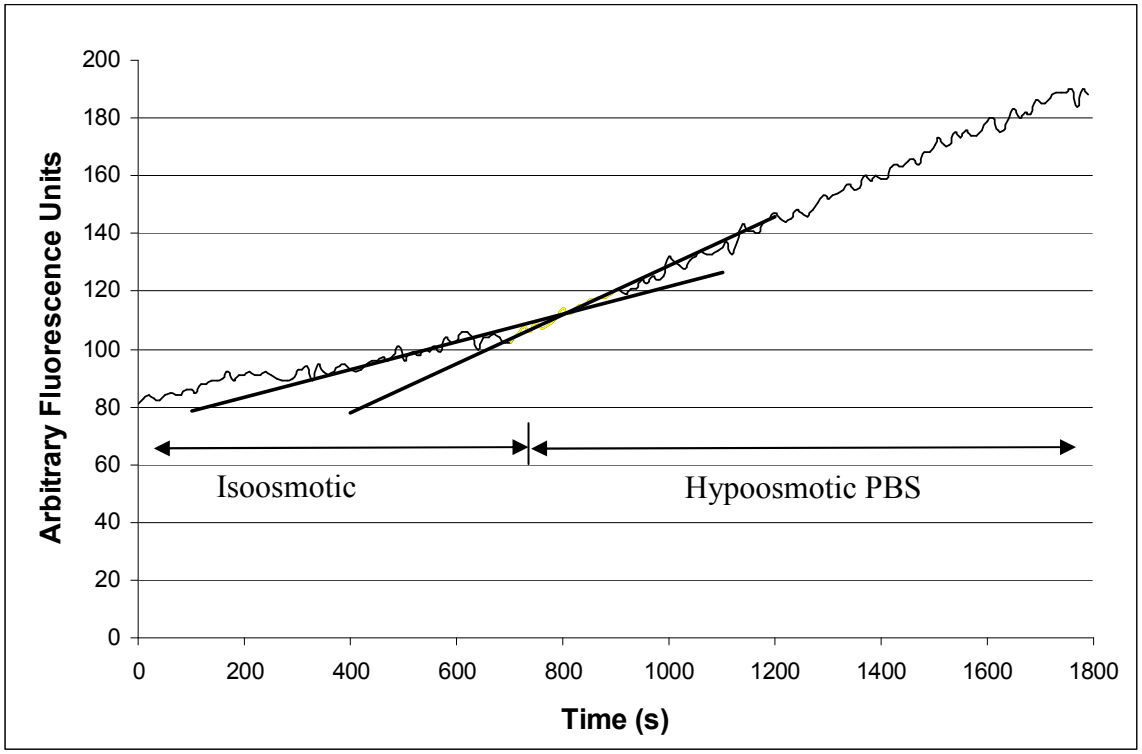

Arbitrary Fluorescence Units
200
180
160
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Time (s)
Isoosmotic
Hypoosmotic PBS

Figure 4b

However, with the addition of carbenoxolone the sudden drops in intensity during solution changes no longer present (Figure 4b).

In figure 4b there was a 60% increase in the rate of change in oxDCF fluorescence that began when the solution was changed to hypoosmotic PBS (150 mOsm) indicating an increase in ROS production rate. To normalize the data for differences in dye loading or cell density, the fluorescence intensity for each data point was expressed relative to the value measured in isoosmotic conditions immediately before hypoosmotic or other experimental solutions were added. The mean of the normalized change in oxDCF fluorescence for 18 trials of cells exposed to 150 mOsm hypoosmotic PBS is presented in Figure 5. The immediate change in ROS production due to hypoosmotic exposure was determined by comparing the slope obtained over one minute prior to the change in hypoosmotic PBS to that obtained during the first minute after the change to hypoosmotic conditions. The blue line represents the linear regression of the mean normalized fluorescence intensities determined during the last minute of isoosmotic PBS perfusion. The yellow line represents the linear regression of the mean normalized fluorescence intensities determined during the first minute of hypoosmotic PBS perfusion. The slope of the mean normalized fluorescence increased from 0.000382 normalized fluorescent units/sec during the last minute of isoosmotic exposure to 0.001143 normalized fluorescent units/sec when the solution was changed to hypoosmotic PBS, an increase of almost 3-fold. On average, cells returned ROS production to 0.000501 normalized fluorescent units/sec during the last min of hypoosmotic PBS treatment.

Figure 5: Hypoosmotic swelling causes a transient increase in ROS production. C6 cells were loaded with DCFDA for 15 min and then were transferred to a fluorescence cuvette for perfusion with 37 ºC isoosmotic PBS containing 100 μM carbenoxolone. Fluorescence intensity determined every 10 s is expressed relative to the value measured at the end of the isoosmotic exposure. The rate of ROS production was measured in isoosmotic PBS for 10 min and then the perfusate was changed to hypoosmotic PBS plus 100 μM carbenoxolone for an additional 10 min. Values are the mean ± SEM of 18 independent measurements. The lines represent a linear regression of the data during 60 s prior to and immediately after the perfusate solution was changed to hypoosmotic PBS.

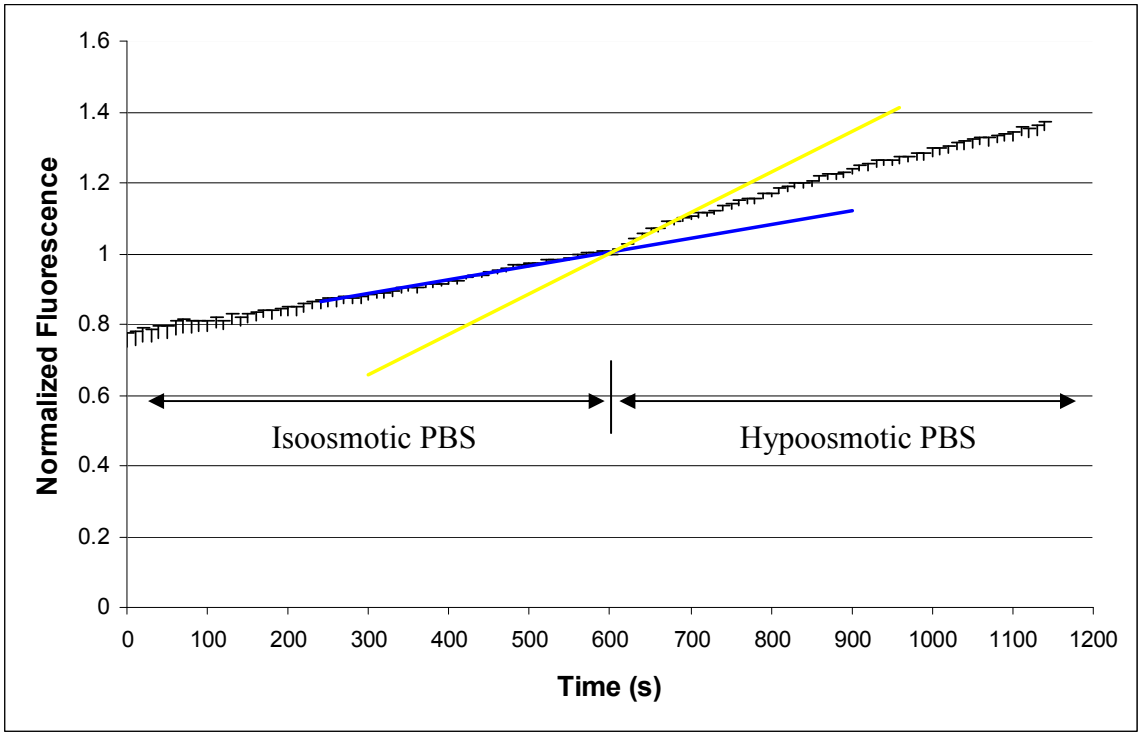

Normalized Fluorescence
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
Time (s)
Isoosmotic PBS
Hypoosmotic PBS

Figure 5

Since the fluorescence cuvette holding the cells was 3 ml and the syringe moved the solution into the cuvette at 1 ml/min, averaging ROS production over 1 min may underestimate the initial change in ROS production due to hypoosmotic exposure. However, a 3 minute time period was chosen to determine ROS production for subsequent analysis to ensure the solution change in the cuvette was complete.

To examine the hypoosmotic dose-dependence of ROS production I measured the increase in ROS production during exposure to either 200 mOsm PBS or 150 mOsm PBS. For both 200 mOsm and 150 mOsm the rate of change in oxDCF fluorescence significantly increased during the initial exposure to hypoosmotic PBS (initial hypoosmotic) (Figure 6). The slope of the oxDCF fluorescence curve during the first 3 min of exposure to 200 mOsm hypoosmotic PBS was 141.6 $\pm$ 18 % of the baseline rate in isoosmotic PBS and for 150 mOsm hypoosmotic PBS the slope was 160.2 $\pm$ 19.6 % of the baseline rate in isoosmotic PBS. The slope for 200 mOsm and 150 mOsm hypoosmotic exposure was very close, suggesting that there was not a dose-dependence for hypoosmotic exposure. In both 200 mOsm and 150 mOsm PBS the rate of ROS production decreased to baseline by the last 3 min of the 10 min exposure period to hypoosmotic PBS.

Inhibitors of ROS Production

Antioxidants were added to see if they could block or reverse the effects of hypoosmotic exposure on ROS production. I first examined the effects of these drugs during isoosmotic exposure. One of two antioxidants, vitamin E (trolox C (150 μM)) and vitamin C (ascorbate (400 μM)) were added to the perfusing isoosmotic PBS after the

Figure 6: The magnitude of increased ROS production rate in hypoosmotic conditions. C6 cells were loaded with DCFDA for 15 min and then were transferred to a fluorescence cuvette and perfused with 37 ºC isoosmotic PBS containing 100 μM carbenoxolone. Rate of ROS production was measured in isoosmotic PBS for 10 min and then perfusate was changed to hypoosmotic PBS (200 mOsm or 150 mOsm) for an additional 10 min. The rate of ROS production was determined during the first 3 min of perfusion with hypoosmotic PBS (0-3 min) and during the last 3 min of the experiment (7-10 min). These values are normalized to the ROS production rate measured in isoosmotic PBS during the 3 min prior to the start of exposure to hypoosmotic PBS.
Values are the mean ± SEM of 11-18 independent measurements.
* Indicates values that are statistically different than 100%.

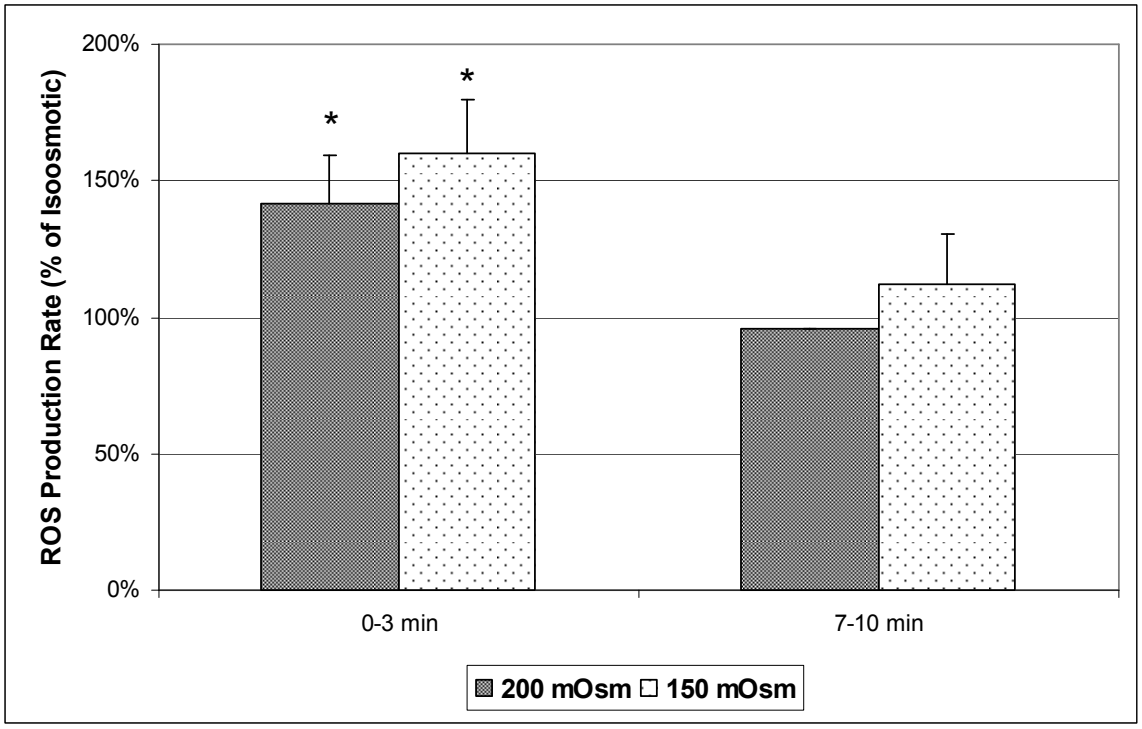

ROS Production Rate (% of Isoosmotic)
200%
150%
100%
50%
0%
*
*
0-3 min
7-10 min
200 mOsm
150 mOsm

Figure 6

cells were in the fluorescence cuvette for 10 min to determine the effects of the antioxidants in isotonic conditions. Trolox C significantly increased ROS production in isoosmotic PBS by 35 $\pm$ 12 % (Table 1) while ascorbate had no effect on ROS production.

When trolox C, but not ascorbate, was present in the PBS solutions there was no significant increase in ROS during hypoosmotic treatment (Figure 7). With ascorbate there was a 55.5 $\pm$ 14 % increase in ROS production during the first 3 min of hypoosmotic exposure, which is similar to the effect in control hypoosmotic conditions (without drug). ROS production then decreased, similar to its response in the absence of drug, back to the baseline value. Trolox C, on the other hand, inhibited the initial increase in production of ROS during hypoosmotic exposure. By the end of the experiments with trolox C there was a significant decrease in the rate of ROS production to 51.3 $\pm$ 14.8 % below baseline.

The two major pathways of ROS production are NADPH oxidase and the electron transport chain (ETC) in the mitochondria. In order to determine if ROS production during hypoosmotic conditions was due to either of these pathways, they were blocked using the pharmacological agents, diphenyleniodonium (DPI), a NADPH oxidase blocker, or rotenone plus oligomycin to block the ETC [2, 84].

In isoosmotic conditions DPI did not alter ROS production compared with baseline (Table 1). However, in hypoosmotic conditions ROS production increased to 162.7 $\pm$ 33.3 % of control levels in the presence of DPI during the first 3 min of exposure. ROS production then decreased significantly below the control levels after 10

Table 1: Antioxidant effects on ROS production in isoosmotic conditions

Drug	ROS Production Rate (% of Control)
Control	100%
150 μM Trolox	135 ± 12 % *
400 μM Ascorbate	98 ± 18%
10 μM DPI	105 ± 36%

C6 cells were perfused with isoosmotic PBS prior to antioxidant exposure. Rates of ROS production were measured during the first 3 min of antioxidant exposure and are normalized to the ROS production rate measured during the 3 min prior to the start of antioxidant exposure (Control).

Values are the mean of 6-9 independent measurements.

* indicates values significantly different from 100% ($p<0.05$).

Figure 7: ROS production during hypoosmotic treatment in the presence of antioxidants. Rates of ROS production were measured while cells were perfused with isoosmotic PBS for 10 min and then changed to perfusion with hypoosmotic PBS for an additional 10 min. All solutions contained 100 μM carbenoxolone. For experimental studies, the antioxidants, trolox C (150 μM) or ascorbate (400 μM), were added during the last 5 min of isoosmotic exposure and throughout the hypoosmotic exposure period. The rate of ROS production was calculated during the 3 min immediately after the start of hypoosmotic treatment (0-3 min) and during the last 3 min of hypoosmotic treatment (7-10 min). These values are expressed relative to the rate of ROS production measured in isoosmotic PBS during the 3 min just prior to the start of hypoosmotic exposure (Control). Values shown are the mean ± SEM of 7-18 independent measurements. * indicates significant differences from the rate of ROS production in isoosmotic PBS ($p<0.05$).

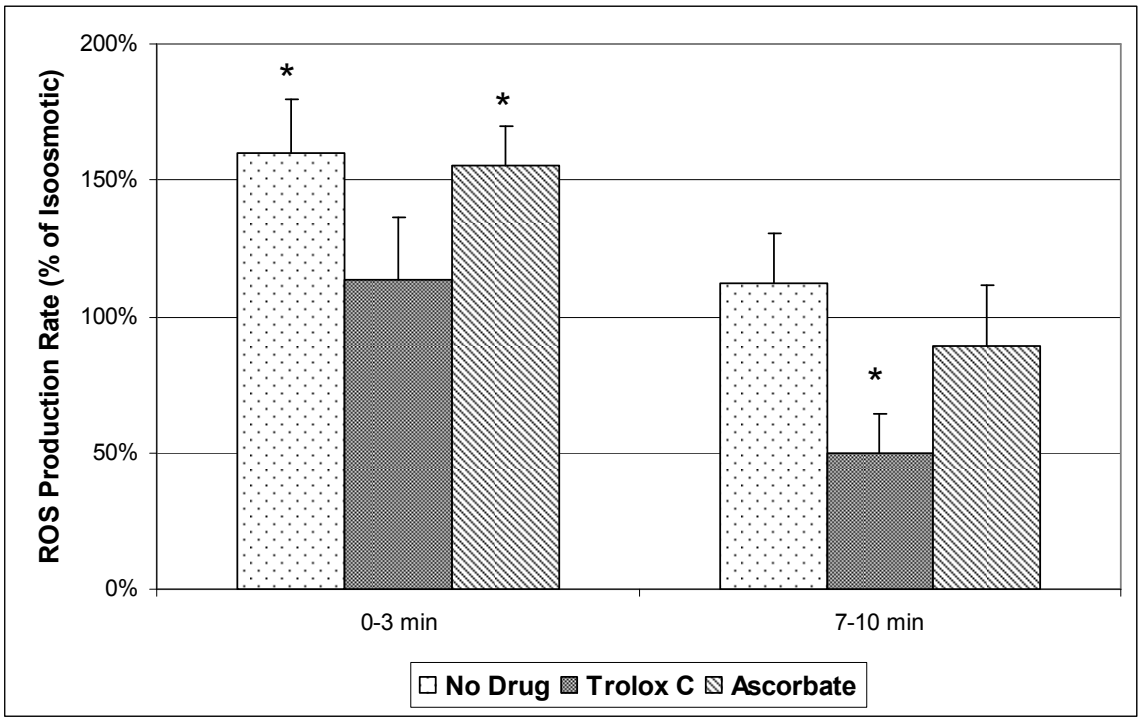

200%
150%
100%
50%
0%
ROS Production Rate (% of Isoosmotic)
*
*
*
0-3 min
7-10 min
No Drug
Trolox C
Ascorbate

Figure 7

min. In the presence of rotenone plus oligomycin there was a significant increase in ROS production throughout the period of hypoosmotic exposure. The initial increase was 83.7 $\pm$ 27.9 %, above control levels, a value which was not significantly different from that measured in cultures exposed to hypoosmotic PBS without drug. But, there was a further increase to 102.6 $\pm$ 36.7 % above the baseline rate during the last 3 min of the 10 min exposure. This was significantly higher than the rate of ROS production measured in control conditions (Figure 8).

Activation of ROS production by ATP:

Because ATP is released from swollen cells and has been shown to activate volume regulation in many cell types, I examined whether elevated extracellular ATP signaled an increase in ROS production in the C6 cells. As described in methods C6 cells received a series of solution changes that mimicked the protocol used when performing fluorescent measurements of ROS production. In the absence of carbenoxolone in PBS, C6 cells showed a significant decrease in ATP efflux in hypoosmotic conditions compared with the efflux measured from cells in isoosmotic PBS (Figure 9). In the presence of carbenoxolone, there was no change in ATP efflux in hypoosmotic conditions compared to the efflux measured in isoosmotic conditions. The assay used to measure ATP efflux in this experiment is relatively sensitive. Cells contain about 3-5 mM of ATP, and the C6 cells effluxed about 0.35 mM of ATP when exposed to PBS in the presence of carbenoxolone. Thus, I was able to measure a efflux of about 10% of the ATP within the cells during the 10 min exposure in C6 cells when exposing them to PBS.

Figure 8: Swelling-induced ROS production during hypoosmotic treatment in the presence of NADPH oxidase or mitochondrial electron transport inhibitors. Rates of ROS production were measured while cells were perfused with isoosmotic PBS for 10 min followed by perfusion with hypoosmotic PBS for an additional 10 min. For experimental cultures, the drug treatments, DPI (10 μM) or rotenone (5 μM) plus oligomycin (10 μM), were added for 5 min prior to and throughout the exposure to hypoosmotic PBS. The rate of ROS production was calculated during the 3 min immediately after the start of hypoosmotic treatment (0-3 min) and during the last 3 min of hypoosmotic treatment (7-10 min). These values are expressed relative to the rate of ROS production measured during the 3 min just prior to the start of hypoosmotic exposure. Values shown are the mean ± SEM of 7-18 independent measurements.
* indicates significant difference from the rate in isoosmotic PBS, $p<0.05$.

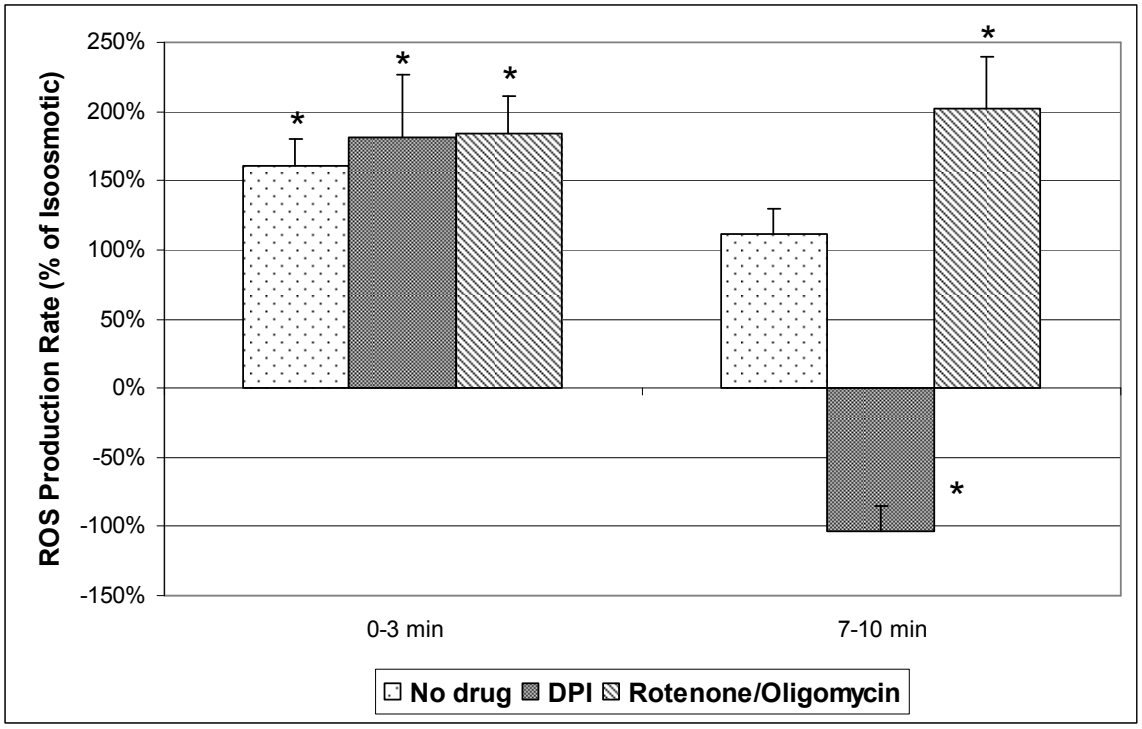

ROS Production Rate (% of Isoosmotic)
250%
200%
150%
100%
50%
0%
-50%
-100%
-150%
*
*
*
*
*
0-3 min
7-10 min
No drug
DPI
Rotenone/Oligomycin

Figure 8

Figure 9: ATP release from C6 cells in isoosmotic and hypoosmotic conditions. Total ATP released from C6 cells was measured during a 10 min exposure to isoosmotic or hypoosmotic PBS. Some cultures had 100 μM carbenoxolone present during the exposure period. For cells exposed to carbenoxolone in hypoosmotic PBS, this drug also was added to the isoosmotic PBS 5 min prior to introduction of hypoosmotic PBS. Values shown are the mean ± SEM of 7-18 independent measurements.

* indicates significant difference from the rate in isoosmotic PBS, $p<0.05$.

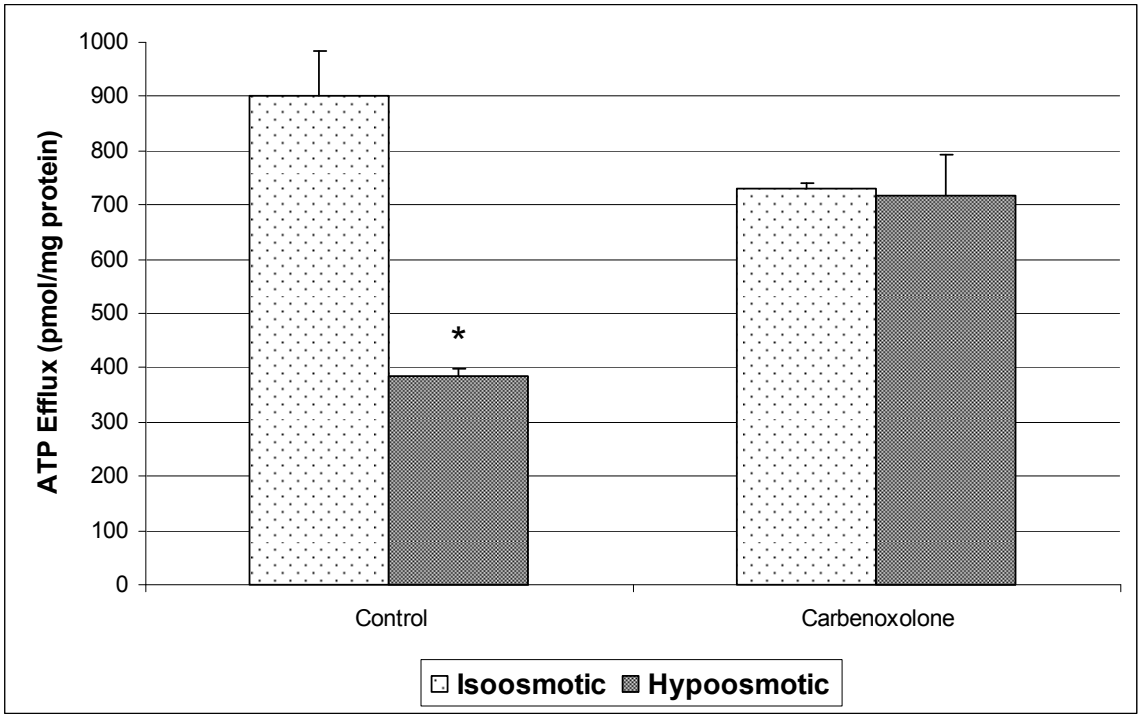

1000
900
800
700
600
500
400
300
200
100
0
ATP Efflux (pmol/mg protein)
*
Control
Carbenoxolone
Isoosmotic
Hypoosmotic

Figure 9

Table 2: ATP does not activate ROS production in isoosmotic PBS

Time	ROS Production Rate (% of Isoosmotic)
0-3 min	1.06 $\pm$ 0.089 %
7-10 min	0.87 $\pm$ 0.077 %

Cells were exposed to isoosmotic PBS for 10 min and then changed to isoosmotic PBS plus ATP for an additional 10 min. The rate of ROS production was calculated during the 3 min immediately after the start of isoosmotic treatment plus ATP (0-3 min) and during the last 3 min of isoosmotic treatment plus ATP (7-10 min). These values are expressed relative to the rate of ROS production measured during the 3 min just prior to the start of ATP exposure.

To test if ATP directly activated ROS production, I added 100 μM ATP to the solution perfusing the cells in isoosmotic PBS (Table 2). This concentration of ATP is in the range that activates channels associated with cell volume regulation (10-100 μM) [98]. ROS production did not increase when ATP was added to cells in isoosmotic PBS. Furthermore, when I added this concentration of ATP to cells perfused with hypoosmotic PBS, ROS production increased similarly to that observed for cells perfused with hypoosmotic PBS in the absence of ATP (Figure 10). However, at the end of the experiment, cells in hypoosmotic PBS plus ATP still had a rate of ROS production that was significantly greater than the rate measured in isoosmotic PBS.

Cytotoxicity:

Apoptotic and necrotic cell death was evaluated following hypoosmotic exposure using annexin V and trypan blue staining and release of lactate dehydrogenase. Because trypan blue only stains cells with permeable membranes, staining with this dye was used to identify necrotic cell death (Figure 11, Table 3). When the cells were in isoosmotic PBS plus trolox C, ascorbate, or DPI there was a very small but significant increase in cell death compared to the amount determined in cells without drug exposure. In hypoosmotic conditions, a significantly greater number of cells were trypan blue-positive compared to the number in isoosmotic conditions. There also was a significant increase in cell death when the cells were exposed to trolox C and ascorbate. This increase was not apparent when the cells were exposed to DPI, though it was close to statistical significance. After 24 hr in H_2O_2 nearly 9 % of the cells were trypan blue-positive. In order to make sure that the cytotoxic cells were not coming off the dish, the cells were

Figure 10: ATP activates ROS production in hypoosmotic PBS. C6 cells were exposed to ATP (100 μM) while they were perfused with hypoosmotic PBS. The normalized rate of ROS production was calculated during the first 3 min of hypoosmotic exposure (0-3 min) and during the last 3 min of the experiment (7-10 min). Values shown are the mean ± SEM of 8-10 independent measurements.

* indicates significant difference from the rate in isoosmotic PBS, $p<0.05$.

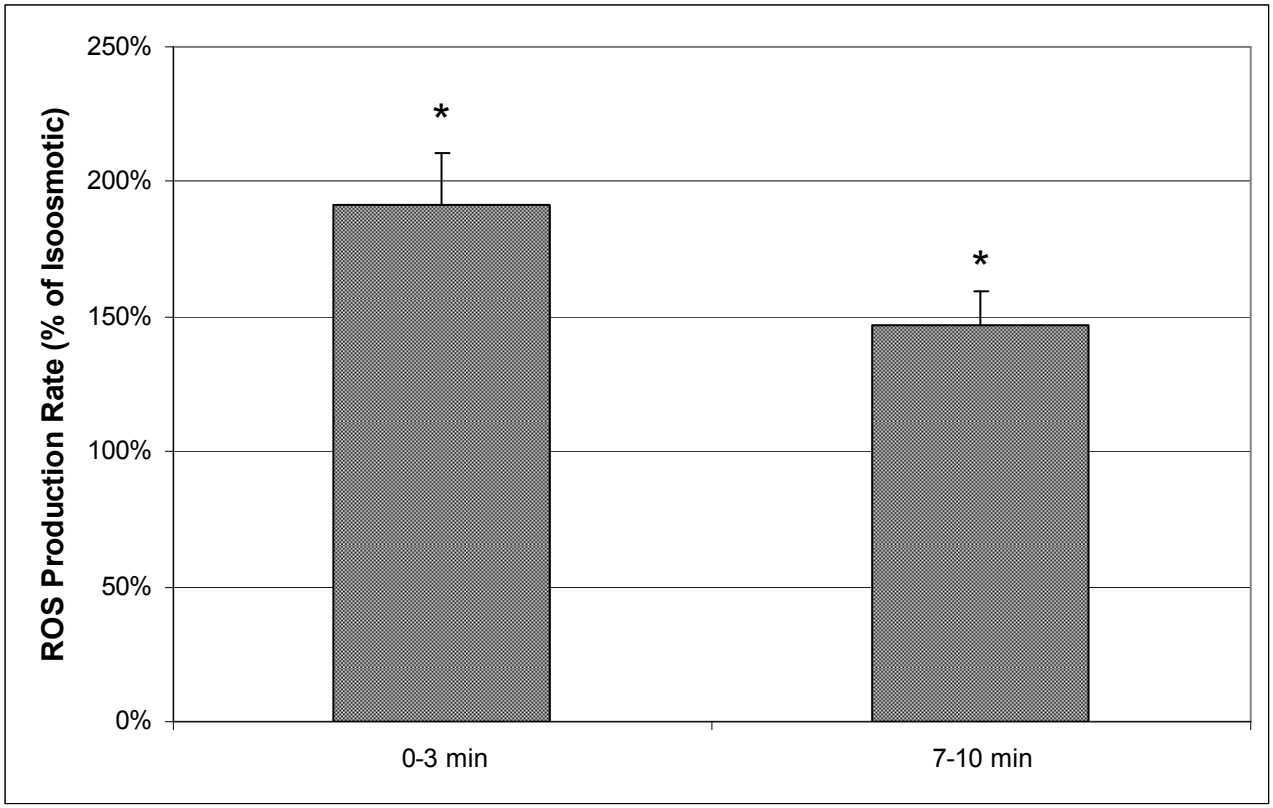

ROS Production Rate (% of Isoosmotic)
250%
200%
150%
100%
50%
0%
*
*
0-3 min
7-10 min

Figure 10

Figure 11: Hypoosmotic-induced cell death. After treatment for 1 hr with isoosmotic PBS, hypoosmotic PBS, or H_2O_2, cells were returned to serum-free growth medium. Twenty-four hours later cells were stained with trypan blue to measure cell death and DAPI to count the total cell number. The bar indicates 100 μm.

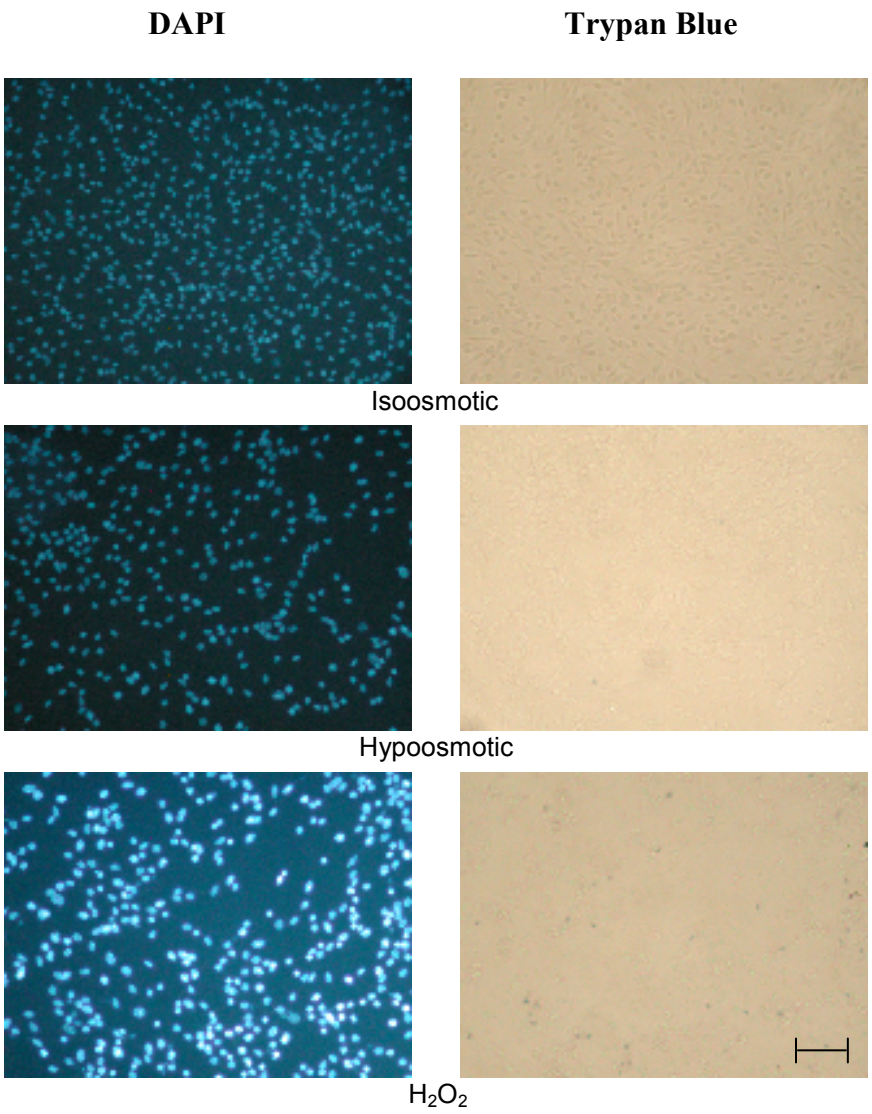


Figure 11

Table 3: Hypoosmotic-induced cell death.

PBS	Treatment	Percent Trypan Blue-Positive Cells
Isoosmotic	None	0.11 ± 0.03 %
	Trolox	0.39 ± 0.05 % *
	Ascorbate	0.35 ± 0.07 % *
	DPI	0.37 ± 0.06 % *
Hypoosmotic	None	0.24 ± 0.05 % *
	Trolox	0.40 ± 0.05 % *
	Ascorbate	0.28 ± 0.04 % *
	DPI	0.22± 0.08 %
Isoosmotic	H_2O_2	8.5 ± 1.6 % *

Cells were exposed to isoosmotic PBS or hypoosmotic PBS for 1 hr and then returned to serum-free growth medium for 24 hours. Data represents the percentage of trypan blue-positive cells. Some cultures had 500 μM H_2O_2 added to the growth medium for 24 hours.

* indicates significant difference from the percent of trypan blue positive cells in isoosmotic PBS, $p<0.05$.

stained with DAPI to count the cell nuclei. Counting the cells revealed that each experimental group averaged about 500 cells per microscopic field. The only exception was H_2O_2, where there was only about 300 cells per microscopic field. The number of cells undergoing apoptosis was evaluated by staining the cells with annexin V, to mark apoptotic calls and DAPI, to mark all cell nuclei (Figure 12). Annexin V is not always an effective dye to use on monolayer cells like C6 cells because there is usually high background fluorescence. The C6 cells did not have this high background in isoosmotic or hypoosmotic PBS and thus annexin V could be used to measure apoptosis (Figure 12). The numbers of DAPI-positive and annexin V-positive cells for various experimental conditions are shown in Table 4. Cells exposed to isoosmotic PBS or hypoosmotic PBS (200 mOsm and 150 mOsm) did not show a significant increase in the cells labeled by annexin V compared with cells maintained in growth medium. Exposure of cells to hydrogen peroxide was used as a positive control. I found that there was a significant increase in apoptotic cell death when the cells were exposed to 500 μM H_2O_2 for 24 hrs.

Cytotoxicity was determined by measuring LDH release from cells. LDH release in each experimental condition was expressed as a percentage of LDH released from cells exposed to isoosmotic conditions measured on the same day. A significant increase in LDH release occurred when cells were exposed to either ascorbate or DPI in isoosmotic PBS (Table 5). Exposure of cells to 150 mOsm hypoosmotic PBS resulted in a 13.5 $\pm$ 6.3 % increase in LDH release over that measured from cells exposed to isoosmotic PBS (Figure 13). The antioxidants did not alter the effect of hypoosmotic PBS on LDH release.

Figure 12: Hypoosmotic conditions do not cause apoptosis. Cells were exposed to isoosmotic PBS or hypoosmotic PBS for 1 hr and then returned to serum-free growth medium for 24 hours. Some cultures had 500 μM H_2O_2 added to the growth medium for 24 hours. Cultures were stained with annexin V and DAPI as described in Methods. The bar indicates 100 μm.

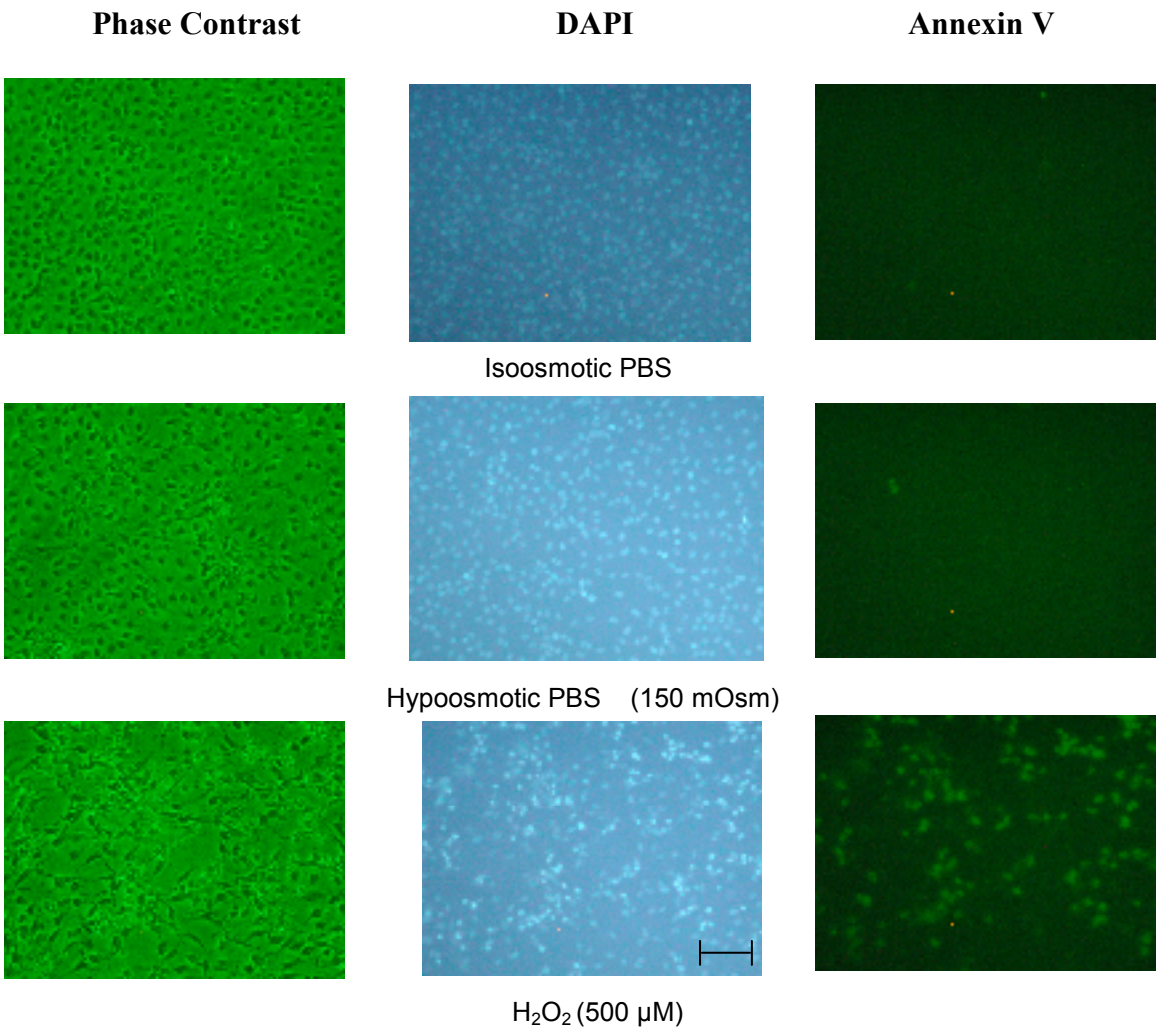

Phase Contrast
DAPI
Annexin V
Isoosmotic PBS
Hypoosmotic PBS (150 mOsm)
H_2O_2 (500 μM)

Figure 12

Table 4: Percentage of annexin V-positive cells does not significantly change following hypoosmotic exposure.

Treatment	Percent Annexin V-positive cells
Control	0.50 ± 0.10 %
Isoosmotic	0.44 ± 0.16 %
Hypoosmotic	0.74 ± 0.14%
H_20_2	50.6 ± 12.5% *

Cells were exposed to isoosmotic PBS or hypoosmotic PBS for 1 hr and then returned to serum-free growth medium for 24 hr. Some cultures had 500 μM H_2O_2 added to the growth medium for 24 hr. Other cultures were not exposed to any experimental condition (control). Cultures were stained with annexin V and DAPI as described in Methods. Values are the mean percentage of annexin V-positive cells from independent measurements.

* indicates values that are significantly different from the percentage of annexin V-positive cells in control cultures.

Table 5: C6 cell cytotoxicity in response to antioxidant treatments.

Treatment	**LDH Release (Percent of Control)**
Control	100%
Trolox C	103.7 ± 4 %
Ascorbate	125.3 ± 13.2% *
DPI	126.2 ± 21.2%*

Cells were exposed to isoosmotic PBS for 1 hr and then returned to serum-free growth medium for 24 hours. Some PBS solutions also contained trolox C (150 μM), ascorbate (400 μM), or DPI (10 μM). LDH content in the growth medium was determined as a fraction of the total LDH in cells plus medium. The fraction of LDH released from cells treated with drug is expressed as a percentage of the fraction of LDH release determined from dishes treated with isoosmotic PBS on the same day (Control). Data are the mean ± SEM for 5-10 independent experiments.

* indicates mean values which are significantly different from the fraction of LDH release measured for cells treated with isoosmotic PBS without drug, $p<0.05$.

Figure 13: Hypoosmotic swelling causes cytotoxicity. Cells were exposed to hypoosmotic PBS (150 mOsm) for 1 hr and then returned to serum-free growth medium for 24 hours. Some PBS solutions also contained trolox C (150 μM), ascorbate (400 μM), or DPI (10 μM). The fraction of LDH released from cells treated with hypoosmotic PBS with or without added drug is expressed as a percentage of the fraction of LDH release determined from dishes treated with isoosmotic PBS without drug on the same day (Control). Data are the mean SEM for 5-10 independent experiments.

* indicates mean values which are significantly different from the fraction of LDH release measured for control cells, $p<0.05$.

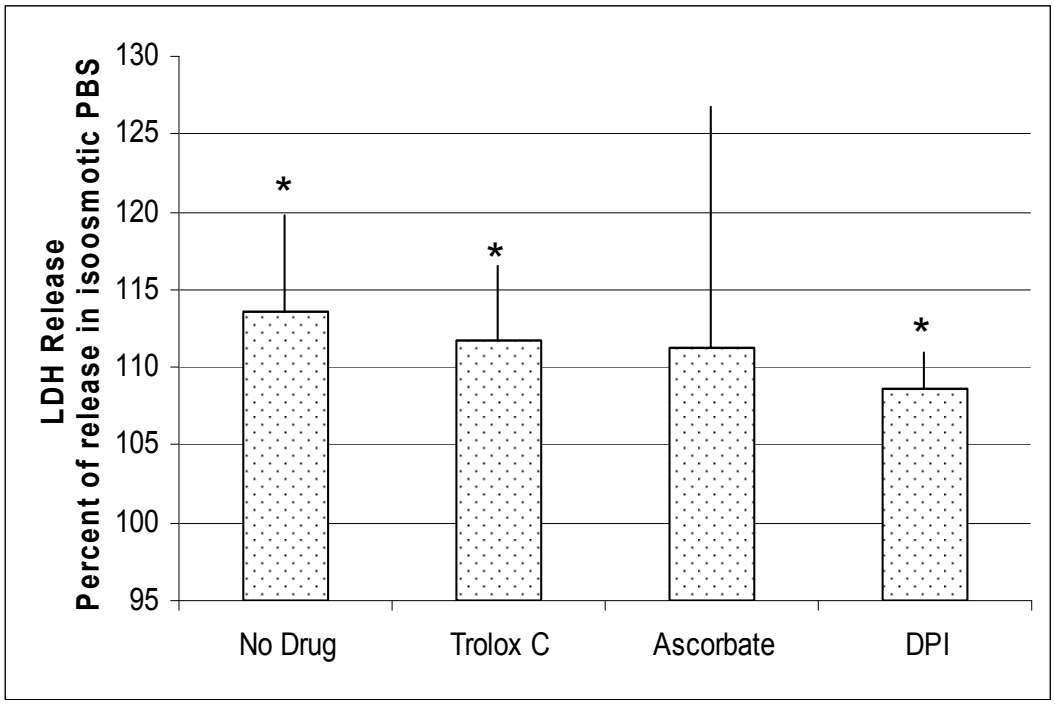

LDH Release
Percent of release in isoosmotic PBS
130
125
120
115
110
105
100
95
*
*
*
No Drug
Trolox C
Ascorbate
DPI

Figure 13

V. Discussion

The results of these studies show that when exposed to hypoosmotic conditions C6 cells increase their rate of ROS production. The hypoosmotic induced increase in ROS production can not be inhibited by blocking NADPH oxidase with DPI or inhibiting the ETC with rotenone plus oligomycin. Antioxidants also were unable to block the increased ROS production during hypoosmotic exposure. When ATP was added to the extracellular solution, C6 cells did not increase their ROS production. Also, there was no increase in ATP efflux under experimental conditions when cells were exposed to hypoosmotic PBS. There was an increase in cytotoxicity when the cells were exposed to hypoosmotic conditions, but this increase was very minimal.

Optimal conditions for measuring oxDCF fluorescence in C6 cells

To determine the characteristics of oxidized DCF (oxDCF) when it is present in C6 cells, I examined excitation and emission spectra of the dye after loading the cells using the diacetate derivative of DCF. Oxidized DCF in solution has a sharp emission peak at 529 nm when excited at 488 nm [12]. The emission spectrum measured for the C6 cells (Figure 3) is very similar to the emission spectrum published by manufacturer (InVitrogen) [1]. Thus, the fluorescence characteristics for oxDCF in the cell are similar to those determined for the dye in solution.

When the oxDCF signal is low, the water Raman signal and scattered light from the excitation beam may interfere with the fluorescence signal. The maximum of the emission spectrum for oxDCF is 529 nm, and as seen in Figure 2, the Raman spectrum obtained with a 470 nm excitation may interfere with the oxDCF spectrum. Using an

excitation wavelength of 475 nm decreased interference of the Raman signal by moving it farther away from the oxDCF spectrum. Moving the excitation wavelength to 475 nm also minimally increased the amount of scattered light from the excitation beam that was detected when the emission light was gathered at 525 nm. Therefore, an optimum was found empirically at 475 nm where the Raman emission is moved as far as possible to longer wavelengths consistent with only a small degree of scattered excitation light appearing in the emission beam.

With an excitation wavelength of 475 nm and emission wavelength of 525 nm, the emission spectrum appeared to contain a sloping background that was possibly due to scattered light from the excitation beam or autofluorescence from the cells. This background signal decreased with increasing wavelength and appeared independent of dye oxidation. At the beginning of the experiment, when the dye levels were low, this background constituted a significant portion of the total signal, and thus, could mask changes in oxidized dye content of the cells. The intensity of the background slope at the maximum dye emission wavelength was estimated by linear regression of fluorescence intensities measured at wavelengths above and below the oxidized DCF emission maximum. The calculated background was then subtracted from the emission peak at 525 nm (Figure 3).

Initial studies suggested cells were losing significant quantities of oxidized dye when solutions were changed. Many cell lines have plasma membrane connexin hemichannels which may be permeable to small molecules such as native and oxidized DCF. These pathways may be activated by cell swelling [136]. Thus, to decrease this loss of dye when changing solutions, an inhibitor of connexin hemichannels,

carbenoxolone, was added to all solutions perfusing the C6 cells. With carbenoxolone present in the perfusion solutions, I did not observe a sudden drop in fluorescence at the time of solution changes as seen previously and the variability of the fluorescence signal during constant perfusion conditions was diminished. C6 cells grown in serum-containing medium do not express a high concentration of connexins [33] typical of mature astrocytes. However, transferring the cells to serum-free medium as was done in these studies, morphologically changes the cells to be more astrocytic in appearance [14]. This differentiation of these cells may increase the amount of connexin expressions since C6 cells treated with serum-free medium plus dimethyl sulfoxide, increase the amount of connexin expression [90]. It is unclear if carbenoxolone is inhibiting oxDCF dye efflux by blocking hemichannels or by some other mechanism.

Hypoosmotic swelling causes increased ROS production

C6 cells produce ROS at a constant rate in isoosmotic conditions. There is a transient increase in the rate of ROS production when cells are exposed to hypoosmotic conditions. The increase in ROS production during hypoosmotic conditions can not be blocked by the antioxidants trolox C or ascorbate, nor can it be inhibited by blocking the NADPH oxidase complex with DPI, or the ETC with rotenone plus oligomycin. It has been shown that other cells increase ROS production during swelling [91, 156, 163]. ROS may be acting as intracellular signals to activate RVD, opening channels for ion efflux and cell shrinkage [156, 163]. C6 cells, like other astrocytic cell lines use a conductive pathway to efflux K^+ and Cl^- during RVD [48, 127, 133]. The way in which

the conductive pathway is activated is still unclear, but specific K^+ and Cl^- channels on the membrane must be activated for RVD to occur in C6 cells.

ROS act as intracellular signals in many processes within the cell by acting on cysteine residues [129]. ROS oxidize the sulfur groups within the cysteine residues forming an intra or inter-molecular bond via the resulting disulfide moiety [37]. For example, when H_2O_2 reacts with the amino terminal of protein kinase C (PKC) there is a conformational change that occurs allowing PKC to be phosphorylated and thus activating the pathway. Contrarily, when ROS reacts with the carboxyl terminal of PKC it is inactivated [129]. PKC has been shown to play a role in volume regulation in rat astrocytes [20]. Inactivation of PKC, which can be accomplished by ROS, induces cell shrinkage [20, 129]. Thus the increased ROS production during hypoosmotic swelling could be used to inactivate PKC in order to reduce cell volume.

The addition of antioxidants during hypoosmotic exposure did not protect the cells from the increase in ROS production. In order to explore the mechanism of ROS production in hypoosmotic conditions, I added two different antioxidants. One was lipid soluble, trolox C, and the other was water soluble, ascorbate. Trolox C actually caused an increase in ROS production during isoosmotic conditions. While this may indicate an increase in cellular ROS production, trolox C also may be shuttling radicals to the DCF. Antioxidants work within the cell by creating stable free radicals that then react with the cellular free radicals to terminate the free radical reactions [158]. A possible reason for the increase in oxDCF when trolox C was added to the cell is that the free radicals formed by the antioxidant were directly oxidizing DCF. Therefore, while the results suggested an increase in ROS production, trolox C may be directly oxidizing the dye thus

mimicking increased ROS production. Another possible reason for the increase in oxDCF when trolox C was added in isoosmotic conditions is trolox C is acting as a pro-oxidant. Many antioxidants act as pro-oxidants when they are present in high concentrations. In isoosmotic conditions the antioxodiant (trolox C) to ROS ratio was very high and therefore trolox C could have been acting like a pro-oxidant.

There did not appear to be a significant increase in ROS production during the initial hypoosmotic exposure above the increase that was already present from trolox C in isoosmotic PBS. The trolox C is shuttling radicals to oxidize DCF or pro-oxidant effect was happening at a maximal rate in isoosmotic conditions, thus there was no change with the addition of hypoosmotic PBS. Therefore, when the cells are changed to hypoosmotic solutions there is no further increase possible for DCF dye oxidation. But there was a decrease in the ROS production with trolox C in hypoosmotic PBS at the end of the experiment compared to trolox C in isoosmotic PBS. This would suggest that as the ROS level increased in the cell, the ratio of trolox C to ROS decreased and trolox C was no long acting as a pro-oxidant in the cells.

The addition of ascorbate did not cause an increase in the rate of ROS production in isoosmotic conditions or hypoosmotic conditions. Ascorbate, like trolox C, can act as a pro-oxidant or antioxidant depending on the concentration within the cell. Ascorbate can react with transition metals and form hydroxyl radicals and lipid alkoxyl radicals [32]. Pro-oxidant effects of ascorbate seem to be due to an increase in extracellular ascorbate, not intracellular, and cause an increase in H_2O_2 production [34]. H_2O_2 is permeable to the membrane and thus could enter the cell and oxidize DCF.

Therefore, ascorbate concentrations used in this study did not seem to have a pro-oxidant effect on C6 cells.

In hypoosmotic conditions, there was a significant increase in ROS production that decreased to baseline within 10 min of hypoosmotic exposure. The results found with ascorbate mimicked the results during hypoosmotic PBS, alone. Ascorbate did not act as an antioxidant in hypoosmotic conditions to decrease the amount of free radicals within the cell. The lack of effect with ascorbate could be due its inability to cross the membrane fast enough to have a protective effect on the cells during this short period of exposure.

The intracellular concentrations of ascorbate are tightly controlled by the cell [34]. Because of the size of ascorbate, it is unable to permeate the plasma membrane [167]. The normal uptake of ascorbate is highly Na^+ dependent using a $2Na^+$:1 ascorbate co-transporter [39, 150]. In hypoosmotic conditions, astrocytes also use a volume-sensitive organic anion channel to efflux ascorbate [39]. Many studies pre-treat the cells with ascorbate in order to allow the cell to actively transport ascorbate across the cell membrane. As cells were not pre-treated with ascorbate in this study, there might not have been adequate time for the ascorbate to enter the cell. If the ascorbate was unable to enter the cell this would explain the lack of antioxidant effect in hypoosmotic conditions. Some ROS producers, such as NADPH oxidase, produce the ROS extracellularly. NADPH oxidase produces superoxide extracellularly where it is converted to H_2O_2 and then crosses the membrane to act as an intracellular signal. The lack of effect of extracellular ascorbate seems to indicate that the increased ROS production during

hypoosmotic swelling was due to an increase in intracellular ROS production, not extracellular ROS production.

Site of hypoosmotic induced increase in ROS production

DCF is oxidized to a fluorescent compound when it interacts with any one of several different ROS molecules [94]. To determine the ROS species that was being produced during hypoosmotic exposure and their source, blockers of ROS pathways were used.

The main ROS producers are the NADPH oxidase complex, mitochondria, xanthine oxidase and nitric oxide synthase. In order to determine if ROS produced during hypoosmotic swelling in C6 cells was due to NADPH oxidase complex, DPI was used to inhibit NADPH oxidase. In astrocytes, activation of NADPH oxidase has been shown to increase ROS production [2, 135]. Astrocytes exposed to hypoosmotic conditions increase serine phosphorylation of $p47^{phox}$ [135] that then causes $p47^{phox}$ to undergo a conformational change that opens up a binding site for interaction with the membrane bound component, $p22^{phox}$. When $p47^{phox}$ and $p22^{phox}$ come together, they bring the other components with them to causes activation of NADPH oxidase and, therefore, production of ROS [57]. DPI acts by irreversibly inhibiting flavoproteins such as the NADPH:O_2 oxi-reductase [15, 155]. In my studies, DPI added to cells exposed to hypoosmotic PBS did not inhibit the initial burst of ROS production, but significantly decreased the amount of ROS produced after 10 min of hypoosmotic treatment. These results suggest that NADPH oxidase is not responsible for the initial increase in ROS when cells are exposed to hypoosmotic conditions. However, the further decrease in

ROS production at the end of the 10 min observation period suggests that ROS production in hypoosmotic conditions is biphasic. The continual production of ROS during hyposmotic exposure could be dependent on an activated NADPH oxidase complex.

Another possible site for ROS production during cell swelling is the ETC of the mitochondria. Rotenone plus oligomycin was used to inhibit the ETC for ROS production in hypoosmotic conditions. Rotenone is added to block normal electron transport and thus, collapses the mitochondrial membrane potential. Oligomycin is used to inhibit the reverse movement of electrons driven by reversal of the ATP synthase [28]. Others have shown that oligomycin plus rotenone could block complex I and V, respectively, of the ETC and significantly decrease ROS production [30].

Since oligomycin plus rotenone added to the PBS did not decrease the initial ROS production to swollen C6 cells, I conclude the ETC is not responsible for this increased ROS production. However, when the ETC was blocked, ROS production did not return to baseline at the end of the experiment, instead it continued to increase, at the increased rate, throughout the experiment.

The increase in ROS production during the initial phase of hypoosmotic exposure was not due to NADPH oxidase nor the ETC. These are two of the primary producers of ROS within the cell, but they are not the only ones. Other potential sources of ROS during hypoosmotic swelling are XO and NO synthase. Activation of XO causes an increase in both $O_2^{\cdot -}$ and H_2O_2 [70] which can increase the fluorescence of DCF [94]. Phosphorylation of XO for activation is mediated in part by p38 kinase [81]. This kinase is activated during hyperosmotic stress, and therefore, was not a high priority of

investigation for this study [81]. Nitric oxide synthase is another ROS producer that could be activated during hypoosmotic stress. NO synthase has been shown to be activated in astrocytes during an immune response [61]. NO acts as an intracellular second messenger [58] which could be used in the cells during hypoosmotic swelling to regulate their volume. There is evidence that NO derivatives are able to activate the K-Cl co-transporter [4, 5]. This would indicate that NO possibly plays a role in RVD in some cell lines. C6 cells do not use the K-Cl co-transporter for RVD, and it is still unclear if NO activates the KCl efflux via the conductive pathway, which is the pathway that C6 cells use.

ATP- activated ROS production

Hypoosmotic swelling-induced ATP release is known to occur in many different cell types [74]. Extracellular ATP is used to induce various biological responses via the activation of purinergic receptors through autocrine and paracrine mechanisms. The ATP released into the extracellular space during hypoosmotic swelling is known to stimulate processes that regulate cell volume [27, 71, 74, 98, 138]. ATP released from primary astrocytes undergoing hypoosmotic stress is necessary and sufficient to activate volume-regulated anion channels (VRAC) [86, 113, 114] Li and Olson also found that extracellular ATP activated membrane chloride conductances in astroglial cells and neurons [98]. The presence of extracellular ATP can potentiate VRAC activity in swollen cells which can result in volume regulation by conducting taurine and potentially other small organic molecules [98]. The organic and inorganic osmolytes that are lost from the cell via VRAC result in volume regulation [113].

Volume-regulated anion channels are an important part of volume regulation in several cell types. Two molecules that play a role in the activation of VRAC are ATP and ROS, though it is unclear how these two molecules activate VRAC [114, 163]. Extracellular ATP activates P2Y receptors causing the release of Ca^{2+} from intracellular stores via activation of a phospholipase C (PLC) signaling pathway [114]. The intracellular Ca^{2+} activates PKC and calmodulin as parallel pathways. Calmodulin activates Ca^{2+}/calmodulin-dependent kinase II which along with PKC regulates VRAC activity [114]. Other studies have shown that H_2O_2 produced via increased NADPH oxidase activity is necessary to activate volume sensitive Cl^- channels [114, 163]. However, the relationship between ATP and ROS pathways for activation of VRAC channels is not clear.

ATP efflux is common among many mammalian cells and is induced by stimuli such as changes in cell volume, mechanical stress, or cAMP [74, 113]. When most mammalian cells are swollen under hypoosmotic conditions they release ATP from within, and thus operate as an autocrine activator of volume regulated channels such as VRAC [113]. Contrarily, in my experiments the efflux of ATP from C6 cells was not increased under experimental conditions when carbenoxolone was added during hypoosmotic exposure. This suggests that ATP is not likely to play an important role in volume regulation of C6 cells.

ATP did not increase ROS production in isoosmotic conditions and thus, I conclude increased extracellular ATP alone is not sufficient to activate ROS production. The lower concentration of ATP, such as 100 μM that was used in this experiment, is able to activate P2Y-receptors which participate in volume regulation in hypoosmotic-

medium [86]. Millimolar concentrations of ATP are needed to activate the $P2X_7$ receptor which is responsible for ATP-induced cell swelling and necrotic cell death [79]. Most cells have ecto-ATPases in the extracellular space that hydrolyzes extracellular ATP to AMP. In C6 cells, the primary ecto-enzyme is ecto-nucleotide pyrophosphatase [38, 79]. When ATP is added to the extracellular space of C6 cells, some ATP is hydrolyzed to AMP and PP_i. When the extracellular concentration of ATP is from 1-10 μM the ecto-enzymes of C6 cells are about 75% effective at hydrolyzing ATP to AMP. At higher concentrations such as 100 μM the ecto-enzyme is only about 50% effective [38]. Therefore, it is possible that about 50% of the ATP that was added to the cells was hydrolyzed to AMP and PP_i before it reached the cell membrane. In hypoosmotic conditions with ATP there was a significant increase in ROS production similar to that observed in the absence of exogenous ATP. However, in hypoosmotic conditions plus ATP the increase in ROS production continued throughout the experiment, remaining elevated for as long as 10 min. This suggests that there is a synergistic interaction between swelling and ATP to enhance ROS production similar to Kimelberg's and Mogin's data on amino acid release and Li et al.'s data on anion channels [86, 98, 114]. Mogin and Kimelberg found that ATP release is not necessary or sufficient to activate VRAC, but ATP adjusts the already active VRAC [114]. Similar to this study, Li et al. observed that ATP enhanced the activity of amino acid efflux through anion channels during hypoosmotic swelling [98]. The current data suggests that ATP is not sufficient to activate ROS production, but that ATP does enhance ROS production. ATP would provide a positive feed-forward loop that increases ROS production explaining the continual increase in ROS production at the end of the 10 min exposure.

Cytotoxicity:

Cell death can occur in two different ways, necrosis and apoptosis. ROS can play a part in inducing both forms of cell death. In C6 cells, apoptosis is mediated by p53 and ERK. Activation of these pathways can be initiated by H_2O_2 [24, 35, 87, 96]. ROS can induce necrosis when there is an uncontrollable increase in ROS. In these situations, ROS are no longer acting as second messengers within the cell; instead, they are oxidizing DNA, proteins, and lipids. The oxidation of DNA, proteins, and lipids causes irreversible cellular damage [106, 130].

Using 3 methods to measure cell death I found that there was a slight increase in cell death via necrosis due to hypoosmotic exposure. Trypan blue staining revealed there was a significant increase in necrotic cell death when the cells were exposed to hypoosmotic PBS compared to isoosmotic PBS. There was a 2-fold increase in cell death when the cells were exposed to hypoosmotic conditions, but this increase in cell death was only about 0.24% of the total cell number.

The antioxidants trolox C and ascorbate as well as the NADPH oxidase inhibitor DPI increased the amount of cell death measured in cells exposed to in isoosmotic PBS. While there was a 3-fold increase in cell death when the cells were exposed to trolox C, ascorbate, and DPI, this increase was very small; from 0.11% in isoosmotic cells to 0.35% – 0.39% in treated cells.

The increase in cell death observed in C6 cells exposed to hypoosmotic conditions could not be prevented with the antioxidants trolox C or ascorbate. Blocking NADPH oxidase with DPI also did not protect the cells. While it is possible that dead cells came off the dish and therefore were not stained with trypan blue, the DAPI staining seemed to

suggest otherwise. Each of the experimental groups had the same density of cells per microscopic field. In contrast, cells exposed to H_2O_2 had a significantly lower count of cells per microscopic field.

I determined if cell death increased due to apoptosis in hypoosmotic conditions using annexin V staining. There was no significant increase in the cell death via apoptosis when the cells were exposed to hypoosmotic or isoosmotic medium. Hydrogen peroxide, used as a positive control, gave a significant increase in cell death.

To determine if necrosis occurred when the cells were exposed to hypoosmotic conditions we measured the LDH release of the cells. When the cells were exposed to hypoosmotic PBS there was a significant increase in cell death, but the increase in cell death was only 13.5 ± 6.5 % above control levels. This suggests that hypoosmotic treatment did have an effect on the cells, but it did not cause wide spread cell death throughout the culture. When cells were exposed to ascorbate or DPI in isoosmotic PBS there was a significant increase in cell death. In isoosmotic conditions, trolox C did not induce an increase in cell death.

The cell death measured in the current experiment was not due to an increase in ROS production. When cells that were treated with ascorbate were exposed to hypoosmotic solutions they did not increase their ROS production, never-the-less there was a significant increase in cell death. If the cell death was due to the increased ROS production, then cells that did not increase in ROS production should not have increased in cell death. Astrocytes, like many other cell types, have endogenous antioxidants that may protect them from damage caused by the increase in ROS production that occurs when the cells are swollen in hypoosmotic conditions [145]. It is probable that the

antioxidants within the C6 cells were capable of preventing cell death due to the increased ROS production. Since the increase in cell death did not correlate with the ROS production, the cell death observed in these experiments must be due to another mechanism within the cell.

The increase in LDH measured in hypoosmotic treated cells was quantified different from cell death determined with trypan blue staining. There was a statistically significant increase in cell death with both LDH released and trypan blue staining, but the percent of cell death with trypan blue was much lower than the 12% increase measured for LDH release (only about a 0.25% increase in hypoosmotic exposure over that of control cells). The much lower percentage of cell death in response to hypoosmotic exposure measured with the trypan blue staining could be because the LDH is a more sensitive indicator of cell injury than trypan blue. However, though the results are not quantatively comparable, the trends are similar. There was a small increase in necrotic cell death with hypoosmotic exposure, but this death was not due to an increase in ROS production.

The cell death observed in this study was very low and therefore could be accounted for by daily cellular turnover. Normal turnover for most cell lines in a day is about 10%; therefore, the cell death found in this experiment was very likely due to cell turnover. The increase in cell death, though it is statistically different is not physiologically significant.

VI. Conclusion

The data presented here suggest that C6 cells exposed to hypoosmotic conditions increase ROS production. This ROS production is not directly activated by extracellular ATP release from the cells, but exogenously applied ATP further increased ROS production. Hypoosmotic conditions slightly increase cytotoxicity of C6 cells, but most cells seem to be capable of surviving the oxidative stress, possibly due to endogenous antioxidants that react with the increased oxygen free radicals.

Cells are exposed to hypoosmotic conditions increase ROS production. This increase is not blocked by either of the antioxidants, trolox C or ascorbate. Since trolox C cause an increase in ROS production in isoosmotic conditions it may be acting as a pro-oxidant within C6 cells. These results also suggest the increased ROS production due to hypoosmotic conditions is biphasic. NADPH oxidase blockers could not inhibit the initial burst of ROS production, but did decrease ROS production by the end of the experiment to below the baseline levels. This may be because the initial burst in ROS is due to another pathway, but prolonged ROS production in hypoosmotic conditions is due to NADPH oxidase.

Unlike many other cell lines, C6 cells do not lose ATP when exposed to hypoosmotic conditions. There was a 10% loss of ATP when the cells were exposed to PBS, but this did not change with hypoosmotic exposure. Thus pathways other than the purinergic receptors must be used to activate the efflux of ions and organic osmolytes responsible for RVD.

Though most of the experimental conditions did induce a significant, but small, amount of cell death, this did not seem to be caused by the increase in ROS production.

These studies suggest that C6 cells are capable of surviving in the midst of extreme osmotic changes without causing significant cytotoxic damage. This study did not specifically look at the change in cell volume, but other studies have suggested the role of ROS in cell volume regulation [163]. There is a large increase in ROS production when the cells are exposed to hypoosmotic conditions, but this increase in ROS production does not cause cell injury.

VII. References

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